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Characteristics of the Toxoplasma Neutralizing Antibody.

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The development of a neutralizing antibody against toxoplasma was first reported in experimentally infected rhesus monkeys.[1] It was also pointed out that this antibody apparently does not develop in all species since none was found in rabbits recovering from infection with the same strain of toxoplasma.[1] This antibody was best demonstrated when mixtures of various dilutions of toxoplasma-infected mouse brain suspension and undiluted serum were injected intracutaneously on the back of one rabbit which received simultaneously similar mixtures prepared with Tyrode's solution or normal monkey serum. The effect of this antibody was also evident when such mixtures were injected intracerebrally or intraäbdominally in mice. This antibody was never found in very great titre since the best sera completely neutralized only 10 to 100 minimal skin lesion doses, although it modified the size and character of the lesions produced by the larger amounts of toxoplasma suspension. The occurrence of this neutralizing antibody in a proved human case of naturally acquired toxoplasmic encephalitis was first reported by one of us in 1941.[2]

In the past 2 to 3 years toxoplasma have been proved to be responsible for a variety of morbid manifestations in human beings by demonstrating their presence in certain human tissues and body fluids microscopically and by animal inoculation.[2,3,4] In order to explore the possibilities of diagnosing this infection during life as well as to ascertain its incidence and the various clinical conditions in which it may exhibit itself, it became necessary to investigate the possible usefulness of certain serological reactions. Thus, one wanted to know how soon after infection the neutralizing antibody became demonstrable, how long it persisted, and whether or not its persistence could be interpreted as indicating a continued infection with this protozoon parasite. In an attempt to answer these questions rhesus monkeys were bled before infection with toxoplasma by the intracutaneous and intraäbdominal routes and weekly thereafter. Since at least 4 sera can be tested on the back of a single rabbit, the monkey sera were tested fresh on some occasions and at other times after storage in a refrigerator at about 5°C; a number of sera

1 Sabin, A. B., and Olitsky, P. K., *Science*, 1937, **85**, 336.

2 Sabin, A. B., *J. Am. Med. Assn.*, 1941, **116**, 801.

3 Wolf, A., Cowen, D., and Paige, B. H., *Am. J. Path.*, 1939, **15**, 657.

4 Pinkerton, H., and Henderson, R. G., *J. Am. Med. Assn.*, 1941, **116**, 807.

TABLE I.
Effect of Storage at 4° to 5°C on Toxoplasmic Neutralizing Antibody.

Monkey serum	Time of storage—days after bleeding								
	Fresh	2	5	7	9	13	14	16	21, 28, 35, 40 and 49
408-2†	10 (100)*								0 (0)
409-2	10 (100)								0 (0)
408-2		10 (500)							0 (0)
409-6		10 (100)					10 (—)		0 (0)
410-7		10 (100)							0 (0)
411-7		10 (100)							0 (0)
6-55			100 (500)			0 (0)			0 (0)
8-54			100 (500)			0 (0)			0 (0)
408-6				10 (100)			10 (—)		0 (0)
408-7				10 (—)‡				0 (0)	
409-7				10 (500)				0 (0)	
410-6					10 (—)				0 (0)
411-6					10 (100)				0 (0)
410-5								10 (—)	0 (0)
411-5								10 (100)	0 (0)

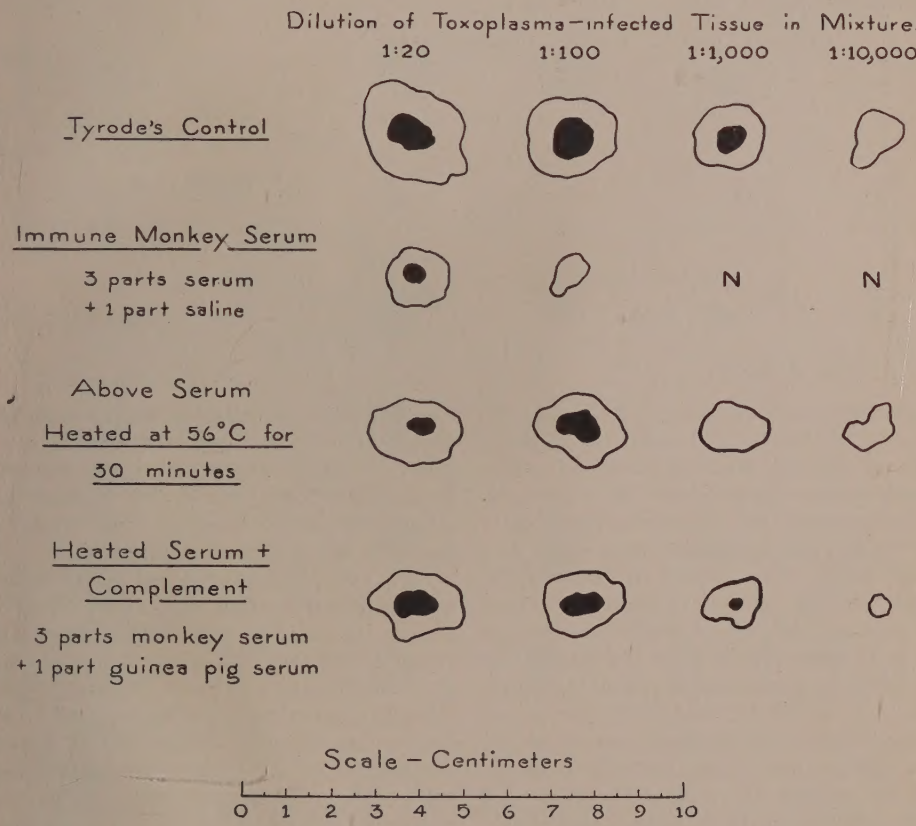
†408-2 Serum of monkey 408, 2 weeks after inoculation with toxoplasma.
*10 (100) Serum neutralized 10 minimal infective skin doses completely and 100 m.i.s.d. partially.
‡10 (—) Serum neutralized 10 minimal infective skin doses completely and had no effect on the other doses.

obtained at different intervals from the same monkey were tested simultaneously on the back of a single rabbit. This method of study at first led us to the erroneous conclusion that this antibody may disappear as early as 6 weeks after inoculation[2] and later it seemed as if the results obtained in this neutralization test were not reproducible. It appeared, however, that when several different sera obtained at weekly intervals from one monkey were tested at the same time the most recent sera regardless of the time after infection, invariably neutralized while those which had been stored in the refrigerator for 2 weeks or longer were usually negative. Furthermore, when a previously positive serum was retested after more than 2 weeks' storage it was almost always negative. When the data on 15 different positive monkey sera which had been tested more than once after different times of storage in the refrigerator were tabulated (Table I), a significant property of the toxoplasmic neutralizing antibody emerged, namely that the antibody, or some part of it, is very unstable even at refrigerator temperature. All the 15 sera which originally had the neutralizing antibody were completely negative after storage in the refrigerator for 21 days or longer; 2 sera were negative on the 13th day and 2 on the 16th day. When it was then found that the neutralizing antibody in a fresh monkey serum was destroyed by exposure to a temperature of 56°C for a half hour, it became necessary to determine whether or not complement was a factor in the reaction and whether or not the neutralizing effect could be restored by the addition of complement to an inactivated serum. The results of such a test shown in Chart I indicate that complement is not a factor (the guinea pig serum used in the test produced complete lysis of sensitized sheep erythrocytes in a dilution of 1:18) and that the toxoplasmic neutralizing antibody is itself so unstable at 56°C. It has proved possible to preserve the antibody by freezing the serum with solid CO_2 and maintaining it in the frozen state in a CO_2 refrigerator or by drying it from the frozen state.

The questions dealing with the time of appearance, persistence, and significance of the neutralizing antibody were then reinvestigated in new rhesus monkeys which were experimentally infected with toxoplasma by the intracutaneous and intraäbdominal routes. It was found (Table II) that, (1) fresh normal monkey sera were entirely devoid of any neutralizing effect, (2) a small amount of the antibody was already detectable one week after inoculation or 5 days after the first fever

Chart I

Effect of Complement on Inactivated Toxoplasmic Neutralizing Antibody



Mixtures injected intracutaneously on the back of a single rabbit

Resulting lesions traced on 7th day

N = no lesion

● = area of necrosis

in the monkey, (3) the amount of neutralizing antibody present at 2 weeks after inoculation persisted without apparent increase or decrease for more than a year (the limit of observation thus far), and (4) the amount of this antibody is so small that it is no longer detectable by this method with serum diluted 1:10. In order to determine whether this persistence of the neutralizing antibody indicated a persistent infection with toxoplasma,

TABLE II.

Time of Appearance and Persistence of Neutralizing Antibodies in Monkeys with Experimental Toxoplasmosis

Wks. after inoc.	Monkey No.								
	281	392	393	408	409	410	411	6	8
Before inoculation	0(0)	0(0)	0(0)						
1	1(10)	1(—)	1(10)						
2	10(100)	10(500)	10(100)	10(500)	10(100)				
3									10(100)
4								10(100)	
5							10(100)		
6				10(100)	10(100)		10(100)		
7					10(500)	10(100)	10(100)		
9				10(—)	10(100)				
12						10(100)	10(100)		
13				10(100)	10(100)				
15				1(100)					
17						10(100)			
20					10(100)				
29		10(100)	100(500)						
45-46					10(100)	100(500)			
54-55								100(500)	100(500)
60					100(500)	100(500)		100(500)	

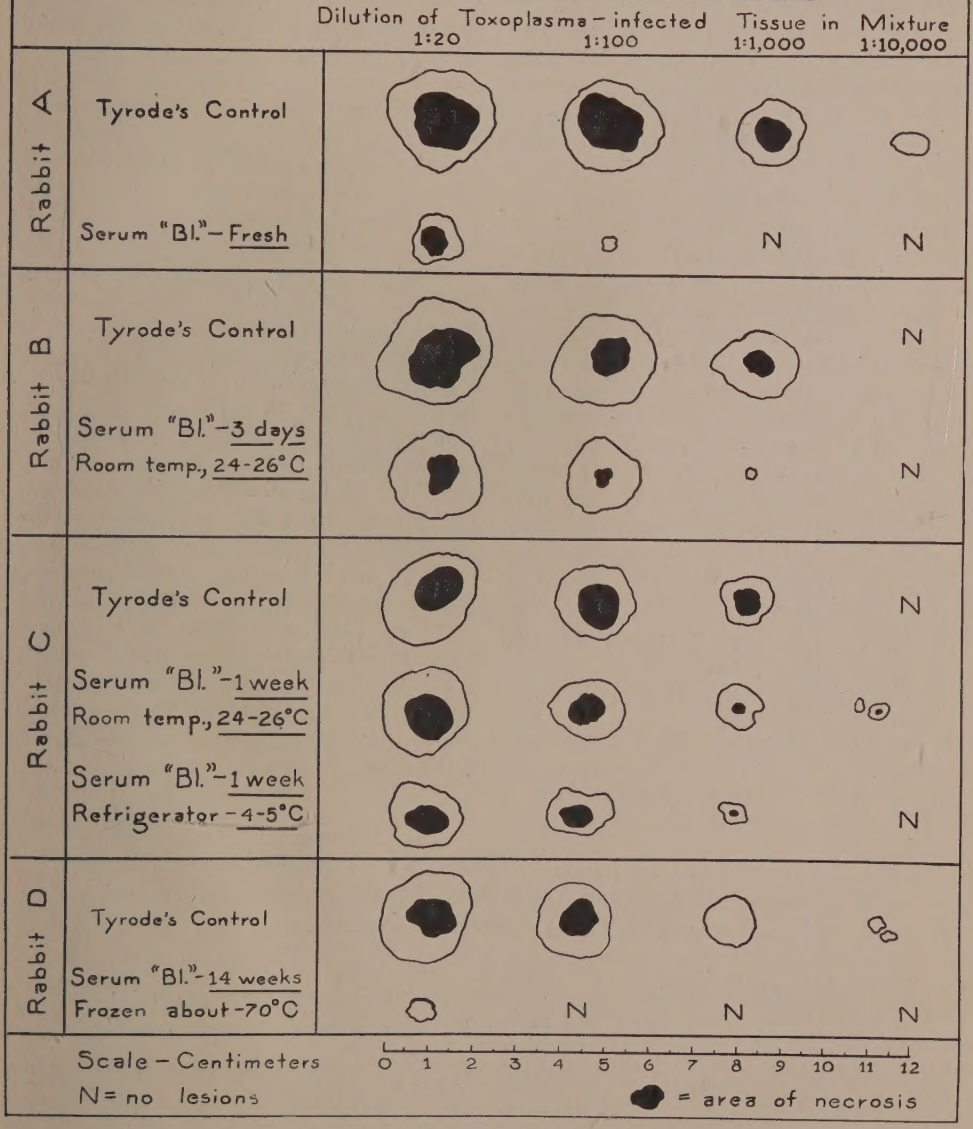
For legends see Table I.

a thorough search was made for the parasite in numerous tissues of 3 convalescent monkeys at various intervals after infection. Rhesus monkeys inoculated with toxoplasma intracutaneously and intraäbdominally usually develop skin lesions and fever which disappear within 10 to 14 days and the animals appear entirely normal thereafter. Rhesus 411 was sacrificed at 14 weeks and Rhesus 408 at 17 weeks after such an inoculation and the following tissues were examined histologically and tested for toxoplasma by intracerebral and intraäbdominal inoculation of mice (each tissue suspension into 4 mice): (1) the axillary and inguinal lymph nodes (corresponding to the inoculated skin sites), (2) spleen, (3) liver, (4) kidneys, (5) adrenals, (6) lungs, (7) brain, and (8) spinal cord. No evidence of persistent infection was found either histologically or by animal inoculation. None of the inoculated mice showed any signs of illness and at the end of one month 2 of each group of 4 mice were sacrificed and the brain and viscera subinoculated into a new group of 4 mice which in turn remained well. This is the most rigorous method that can be used to detect toxoplasma. This procedure was also followed and negative results obtained with the same tissues of Rhesus 392 which died 7 months after inoculation of an adhesive pericarditis, apparently the result of repeated cardiac punctures. *It would appear, therefore, that the persistence of the toxoplasmic neutralizing antibody is not the result of persistent infection with this protozoon parasite.*

Paradoxical behavior of one lot of hyperimmune monkey serum. One of us (A.B.S.) prepared a pool of "hyperimmune" monkey serum in 1935 by bleeding out 4 rhesus monkeys which had received repeated intraabdominal injections of toxoplasma-containing tissues. At first this "hyperimmune" serum neutralized no better than any other convalescent monkey serum. When it was retested in 1939, however, it regularly completely neutralized even the largest concentration of toxoplasma with which it was mixed both upon injection into the rabbit's skin and intracerebrally in mice. This serum has been stored in an ordinary refrigerator for the past 7 years and, contrary to the experience with all the other sera containing toxoplasmic neutralizing antibody, is still as potent as ever. Also contrary to the effect which heating at 56°C for 30 minutes has on all the other toxoplasma-immune sera, this "hyperimmune" serum lost none of its antitoxoplasmic effect after exposure to this temperature. Because of its history and prop-

Chart II

Tests on Single Specimen of Human Toxoplasma Immune Serum Stored under Different Conditions



erties the antitoxoplasmic effect of this "hyperimmune" serum cannot be regarded as being due to the regular neutralizing antibody. At one time before 1939 this serum became overgrown with molds during the course of its storage in the refrigerator and it was sterilized by Seitz filtration and has remained sterile ever since (pH 7.2). Suspecting that the particular mold may have produced an antitoxoplasmic substance, we filtered 5

old, moldy, normal monkey sera and tested them against toxoplasma, but with completely negative results.

Neutralizing antibodies in other animals. Fresh sera of normal rabbits, mice, cats, and dogs contained no antibody nor was any found in these animals after recovery from experimental infection with toxoplasma (the studies on the sera of cats and dogs were carried out in association with Dr. Joel Warren).

Lability of toxoplasmic neutralizing antibody in human sera. Although a number of scattered tests had indicated that the toxoplasmic neutralizing antibody was probably as labile in human as in monkey sera, it was desirable for practical and other reasons to determine some of the limits of this lability. Serum, known to contain neutralizing antibody, was obtained from an 18-month-old infant with congenital toxoplasmosis[5] and divided into several portions. Part was immediately frozen and stored in a solid CO_2 refrigerator, part was tested fresh, and other portions were tested after storage at room temperature (about 24° to 26°C) or in an ordinary refrigerator (about 4° to 5°C). The data illustrated in Chart II reveal that the same serum which yielded strongly positive results when tested fresh or after being frozen at about 70°C for 14 weeks gave an equivocal result after 3 days at room temperature or 1 week in the refrigerator and a distinctly negative result after 1 week at room temperature. The neutralizing antibody in the human serum was also inactivated by heating at 56°C for 30 minutes.

Summary. The toxoplasma neutralizing antibody was found to be so labile even at temperatures of about 5°C, that it could disappear after one to 2 weeks of storage in an ordinary refrigerator. The antibody effect was destroyed by heating at 56°C for 30 minutes and could not be restored by the addition of fresh complement. The antibody could be preserved for months at the low temperature provided by solid CO_2 in an insulated box. Antibody was absent in the fresh sera of rhesus monkeys before infection with toxoplasma but appeared within one to 2 weeks after infection and persisted for at least 14 months which is the limit of observation thus far. This persistence of neutralizing antibody was not associated with persistence of toxoplasma in the monkeys, since none could be found in tests on a large number of tissues from 3 monkeys, 14, 17, and 26 weeks after infection. No neutralizing antibody was found in the fresh sera of normal rabbits, mice, cats, or dogs, nor did these animals develop any appreciable antibody after recovery from experimental infection with toxoplasma.

5 Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 6.

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Toxoplasma Neutralizing Antibody in Human Beings and Morbid Conditions Associated With It.

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By actual demonstration of the organisms in the tissues, by microscopic and animal inoculation methods, toxoplasma have already been proved to be responsible for the following morbid conditions in human beings: (a) congenital encephalomyelitis becoming apparent in the newborn period or *in utero*,[1,2] (b) acute encephalitis in childhood,[3] and (c) a spotted-fever-like syndrome associated with pneumonitis.[4] The fact that mothers who had given birth to infants with proved

1 Wolf, A., Cowen, D., and Paige, B. H., *Am. J. Path.*, 1939, **15**, 657; *Science*, 1941, **93**, 548.

2 Paige, B. H., Cowen, D., and Wolf, A., *Am. J. Dis. Child.*, 1942, **63**, 474.

3 Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

congenital toxoplasmosis, themselves gave no definite history of illness suggested that mild, subclinical, or even inapparent infections with toxoplasma may occur.

When the special properties of the toxoplasma neutralizing antibody were discovered and convincing evidence was obtained that the rabbit skin neutralization test yielded reproducible results,[5] a study was undertaken to determine, (a) the incidence and significance of this antibody in human beings, and (b) whether or not certain morbid manifestations in which a toxoplasmic etiology might be suspected, were associated with a high incidence of positive neutralization tests. The following procedure was adopted for the test:

Animals. Clear-skinned (*i.e.*, without ingrown hair), well-nourished rabbits, weighing about 3 kg gave the best results. The hair was clipped with an Oster animal clipper (000 or 0000). Among the 105 rabbits obtained from 2 different dealers (Ohio and New York) and used for neutralization tests in the past year, we encountered 5 which were naturally immune (this incidentally is an interesting indication of the possible incidence of nonfatal toxoplasmic infection among rabbits in certain regions). A naturally immune rabbit is recognized by the fact that small, rapidly regressing or insignificant skin lesions develop with the control mixtures and the animal survives at a time when other rabbits inoculated simultaneously with the same toxoplasma suspension develop the usual progressive lesions and die.

Serum. Whenever possible the serum was used on the day of bleeding. Sera which had been in the refrigerator overnight or in the mail for 24 to 48 hours (during the winter months) have also yielded positive results. The unused portion of each serum was frozen rapidly in a lusteroid container and stored in an insulated box containing solid CO_2 to permit repetition of the test whenever necessary. Serum which could not be tested within 24 hours of bleeding was similarly frozen and stored in the CO_2 refrigerator.

Toxoplasma suspension. The R. H. strain of toxoplasma, which had undergone many mouse passages since it was isolated in 1939 from a fatal case of human encephalitis,[3] was used in these tests. The brains of mice succumbing 4 days after intracerebral inoculation were examined by means of Wright-stained films to make certain of the presence of large numbers of toxoplasma and the absence of bacteria. At least 2 such brains were ground in a mortar without an abrasive and enough Tyrode's solution was added to make a 10% suspension. This suspension was then allowed to sediment spontaneously for 30 minutes in an ordinary refrigerator. From the opalescent supernatant liquid which is regarded as the 1:10 dilution, the 1:50, 1:500 and 1:5000 dilutions were prepared using Tyrode's solution as the diluent. Mixtures consisting of 0.15 cc of undiluted serum or Tyrode's solution (for the control) and 0.15 cc of each of the above dilutions thus yielded final dilutions of toxoplasma-containing mouse brain suspension of 1:20, 1:100, 1:1000, and 1:10,000. After thorough shaking these mixtures were left at room temperature for 30 minutes before inoculation. The back of each rabbit was marked off into 20 squares with an indelible pencil to accommodate all the dilutions of the Tyrode's solution control and 4 sera. 0.2 cc of each mixture was injected intracutaneously.

Interpretation of results. All human sera produce erythema and oedema in the rabbit's skin within 24 hours, but this reaction has usually disappeared by the 4th day when the toxoplasma lesions begin to appear. The toxoplasma skin lesions are raised, indurated and the ones resulting from the larger concentrations undergo central necrosis; they are fully developed by the 7th or 8th day, when a record is made by tracing on transparent paper the size of each lesion and of the area of necrosis. The skin lesions begin to blanch after the 8th day and the rabbits with very rare exceptions die of a generalized toxoplasmosis between the 9th and 12th days.

With this strain of toxoplasma and with the infected mouse brain suspension pre-

[4] Pinkerton, H., and Henderson, R. G., *J. A. M. A.*, 1941, **116**, 807.

[5] Sabin, A. B., and Ruchman, I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 1.

TABLE I.
Criteria Used in Interpretation of Toxoplasma Neutralization Tests.

Category of unknown serum	Relative size of skin lesions yielded by mixtures containing various dilutions of infected mouse brain suspension			
	1:20	1:100	1:1000	1:10,000
Negative	++++	++++	+++	++ or +
Negative	++++	++++	+++	0
Equivocal (Repeat test)	++++	+++	++ or +	0
Positive	++++ or +++	+++ or ++	0	0
Strong positive	++	0	0	0

pared according to the above directions, distinct lesions appeared in all susceptible rabbits at all sites inoculated with the 1:20, 1:100, and 1:1000 dilutions of the control mixtures while the 1:10,000 dilution produced lesions less regularly but more often than 75% of the times. The mixtures with most of the distinctly negative human sera yielded skin lesions which were somewhat larger than the Tyrode's solution controls. Table I indicates the criteria used for classifying the sera. Repeated tests on the same specimen of a positive serum (preserved in the frozen state) revealed the reliability of these criteria, and tests on repeated bleedings from the same individual gave ample evidence of the reproducibility of the results.

Results of tests on human sera. Toxoplasma neutralization tests were performed on sera from 151 selected individuals. The following considerations guided the selection of individuals: (a) the presence in infants or children of one or more of the manifestations that may now be considered to constitute the clinical tetrad of congenital toxoplasmosis, *i.e.*, (1) hydrocephalus or microcephaly, (2) cerebral calcification, (3) chorioretinitis, (4) disturbances of nervous function such as convulsions, psychomotor retardation, etc.; (b) the mothers and whenever possible other members of the families of such infants and children; (c) mothers who gave birth to hydrocephalic or microcephalic infants who were either stillborn or died shortly after birth; (d) mothers who gave birth to anencephalic monsters; (e) older children and adults exhibiting chorioretinitis of unknown etiology and resembling the type encountered in infants with congenital toxoplasmosis; (f) children who were hospitalized in Cincinnati during the summer and autumn of 1939 because of an encephalitis bearing a clinical resemblance to the proved fatal case of toxoplasmic encephalitis which occurred at that time;[5] (g) certain unexplained encephalopathies; (h) a large miscellaneous group made up of normal individuals, and children and adults taken at random from the hospital wards or tested because they had febrile illnesses of unknown etiology, obscure disturbances of the nervous system, congenital cataracts or other disturbances of the eyes, etc.

The sera of 92 of the 151 individuals were negative. The 59 individuals whose sera neutralized toxoplasma were derived from all the groups listed above. In the group of infants and children presenting various disturbances which used to be ascribed to birth injury, hemorrhage, congenital deformity, etc., it was found that the incidence of neutralizing antibodies was high in the patients and their mothers only when certain clinical manifestations occurred together (Table II). Thus, when cerebral calcification or chorioretinitis in the macular region or both were associated with hydrocephalus or microcephaly or with convulsions and psychomotor retardation, distinctly positive neutralization tests were obtained in 10 of the 13 infants or children and in 8 of 10 of their mothers. Specific toxoplasmic complement fixation reactions[6] were obtained in repeated tests on different specimens of serum from one of the patients (with suspected congenital toxoplasmosis, first group Table II) and his mother who were regularly negative in the neutralization test. By comparison it is important to note that

[6] Warren, J., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 11.

TABLE II.
Toxoplasma Neutralizing Antibodies in Human Beings with Certain Obscure Diseases.

Clinical manifestations		No. of individuals with toxoplasma neutralizing antibodies among: Patients	Mothers of patients
Cerebral calcification and/or chorioretinitis in macular region associated with hydrocephalus, microcephaly and/or convulsions, psychomotor disturbance, etc. Age group—birth to 15 yrs		10/13	8/10*
Hydrocephalus or microcephaly *without* cerebral calcification or chorioretinitis. Age group—9 wks to 5 yrs		0/10	1/4
Convulsions, psychomotor disturbances, etc., *without* cerebral calcification or chorioretinitis Age group—birth to 5 yrs		1/9	1/2
Chorioretinitis of unknown etiology resembling that seen in congenital toxoplasmosis	*Probably congenital* Disturbance of vision first noted at 5 to 6 yrs. Age time of test, 8-14 yrs	4/4	4/4
	Acquired Disturbance of vision first noted at age 15 to 57 yrs. Age at test, 16 to 59 yrs	5/6	—
Mothers who gave birth to hydrochephalic or microcephalic infants stillborn or died soon after birth		—	3/4
Mothers who gave birth to anencephalic monsters		—	3/8

*8 of the 10 individuals tested had the antibodies in their serum.

among 8 children with hydrocephalus and 2 infants with microcephaly but without either cerebral calcification or chorioretinitis in the macular region there was none with neutralizing antibodies. Similarly among 9 infants and children with histories of convulsions beginning soon after birth, psychomotor retardation, or palsies *not* associated with cerebral calcification or chorioretinitis there was but one child with neutralizing antibodies which were also present in the mother's serum. This child had convulsions and fever for several days after birth and subsequently there was some question of retardation of psychomotor development. It would appear, therefore, that, with one exception, the infants and children ranging in age from several months to 15 years, who together with their mothers had toxoplasma neutralizing antibodies, exhibited either *chorioretinitis in the macular region* or *cerebral calcification* or both in addition to the various other manifestations. It may be of interest to note here that on one occasion the serum of the father (1 of 4 tested) and older normal siblings have also been found to contain toxoplasma neutralizing antibodies, suggesting a high incidence of associated familial, mild or subclinical infection.

The finding of toxoplasma neutralizing antibodies in 3 of 4 mothers who had given birth to hydrocephalic or microcephalic infants, who were either stillborn or died soon after birth, was not surprising although this does not necessarily indicate the true incidence of toxoplasmic infection as the causative factor in such conditions. The possibility that toxoplasmic infection early enough in pregnancy might occasionally lead to anencephaly led to a study of the sera of 8 mothers who had given birth to anencephalic monsters. Three of the 8 had neutralizing antibodies for toxoplasma, negative Wassermanns and no hydramnios, 2 had positive Wassermanns and no hydramnios, and the remaining 3 had hydramnios. While this

evidence is suggestive, it cannot be taken as proof that toxoplasmic infection of the fetus may give rise to anencephaly, but further studies are indicated especially on the anencephalic monsters themselves.

Perhaps the most significant new lead, arising from the application of the neutralization test to various morbid conditions in which the pathogenic potentialities of toxoplasma might have been considered, was found in the high incidence of positive tests (9 of 10) among older children and adults presenting a chorioretinitis of unknown etiology resembling (particularly in its predilection for the macular region) that found in infants with proved congenital toxoplasmosis. The possibility that in at least some of these individuals, the chorioretinitis may have been the only important residue of a congenital toxoplasmosis which was not detected until the children had to begin to read, is suggested by the fact that the sera of the mothers of all 4 of these patients had neutralizing antibodies. In at least one family the sera of 2 older, normal siblings had no such antibody. On the other hand, the appearance of poor vision in later life in the other patients suggests an acquired infection.

Among the other individuals whose sera neutralized toxoplasma there were 2 children who in the summer of 1939, at 10 and 12 years of age respectively, had acute encephalitis characterized chiefly by fever, coma, and convulsions; one recovered completely and the other was left with a changed personality and persistent convulsions. There were also 5 individuals, 10 to 69 years of age, with unexplained encephalopathies presenting predominantly severe, persistent headaches, in some associated with hyperostosis frontalis interna. The sera of 2 laboratory workers exposed to toxoplasma were positive. The serum of a 14-year-old boy with an atypical pneumonia from which he recovered in one week following sulfathiazole therapy was in the positive group as were those of several rabbit handlers one of whom also developed agglutinins for *B. tularense*. Although the present data do not permit a final statement regarding the incidence of toxoplasma neutralizing antibodies among various age groups of the population at large, it would appear to be in the range of 10% or less if one excludes the members of the immediate family of individuals with clinical manifestations of toxoplasmosis.

Summary. A standardized procedure for carrying out the toxoplasma neutralization test in rabbits was described. The sera of 59 of 151 selected individuals were positive. A very high incidence of positive tests was found in infants and children presenting psychomotor disturbances with or without hydrocephalus or microcephaly only when these were associated with cerebral calcification or chorioretinitis in the macular region or both. The congenital character of the disease was confirmed by the regular presence of these antibodies in the mothers of the affected children. Chorioretinitis affecting predominantly the macular region, and resembling that seen in cases of proved congenital toxoplasmosis, but occurring in otherwise apparently normal children and adults was associated with neutralizing antibodies for toxoplasma in 9 of 10 individuals. Certain other morbid conditions associated with toxoplasma neutralizing antibodies were also briefly described.

Acknowledgment. The present study was made possible by the coöperation of many individuals in the departments of pediatrics, neurology, neurosurgery (especially Drs. Maltby and Evans), obstetrics, ophthalmology, and roentgenology. Dr. Bronson Crothers of Boston, Drs. Douglas N. Buchanan and C. Anderson Aldrich, of Chicago, Dr. Rustin McIntosh of New York, and Drs. P. M. Levin and H. Moore, of Dallas, Texas, supplied the sera and information on many of the cases of suspected congenital toxoplasmosis. Mrs. Estelle W. Brown, of Cincinnati, suggested and made possible the investigation of the mothers who had given birth to hydrocephalic, microcephalic, or anencephalic offspring. Dr. Douglas N. Buchanan, of Chicago, and Drs. Vail, Strong and Stephenson, of the department of ophthalmology of the University of Cincinnati, supplied the material on the 10 cases of chorioretinitis in older children and adults, and they will describe in greater detail the clinical characteristics of this condition.

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The Complement Fixation Reaction in Toxoplasmic Infection.

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The need for a rapid and simple serological test for toxoplasmic infection prompted the present investigation of the development of complement fixing antibodies. The neutralization test described in the accompanying communications[1,2] has two chief drawbacks: (1) virulent toxoplasma must constantly be maintained by animal passage, and (2) the result of the test is not known for about a week.

Immune monkey sera possessing neutralizing antibodies were available and the first problem was to determine whether or not a toxoplasma-infected tissue could be found which would yield a suspension or extract that would fix complement specifically in the presence of a known immune serum. The data summarized in Table I reveal that the problem was not simple since the tissues which we knew to be richest in toxoplasma content, either gave no fixation (*e.g.*, mouse brain) or nonspecific fixation (*e.g.*, mouse lung), or were unsuitable for other reasons. The antigen finally found to give specific complement fixation with immune monkey sera was prepared in the following manner:

Rabbits weighing approximately 2 kg were given 0.5 cc of a 10% suspension of toxoplasma-infected mouse brain intracerebrally. When the rabbit died (4 to 8 days after inoculation) its entire brain was ground with enough physiological salt solution to make a 10% suspension which was then stored in an ordinary refrigerator for 18 hr.

TABLE I.
Search for Antigen Giving Specific Complement Fixation with Toxoplasma Immune Monkey Serum.

Toxoplasma infected animal	Tissue	Type of extract	Result
Mouse	Brain	0.85% saline, frozen, thawed 5 ×	No fixation
		Frozen with solid CO_2, pulverized, extr. with saline	" "
		Absolute methyl alcohol	Nonspecific fixation*
"	Lung	" " "	" "
		0.85% saline	" "
		Berkefeld "V" filtrate of saline extr.	" "
"	Peritoneal exudate	Whole heparinized, fresh	Anticomplementary
		Absolute methyl alcohol	"
		" ethyl "	Hemolytic
Rabbit	Spleen	Dried *in vacuo*, powder extr. with methyl alcohol	No fixation
		0.85% saline	Nonspecific fixation
"	Liver	" "	" "
"	Brain	Absolute methyl alcohol	" "
		0.85% saline, frozen, thawed 5 ×	*Specific fixation*

*Nonspecific fixation means that extracts of normal and toxoplasma-infected tissue gave same degree of fixation.

1 Sabin, A. B., and Ruchman, I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 1.

2 Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 6.

This suspension was then alternately frozen in a mixture of solid CO_2 and alcohol and thawed 5 times, and centrifuged on an angle centrifuge at about 3,000 rpm for 30 min. The supernatant liquid constituted the antigen. It was stored without preservative both in an ordinary refrigerator and in the frozen state in an insulated box containing solid CO_2, and retained its activity for at least 3 months. The control antigen was prepared in the same manner using normal rabbit brain.

Procedure of the Complement Fixation Test. The immune sera were inactivated at 56°C for 30 min and used undiluted, 1:5, 1:10, 1:20, etc., or diluted, 1:2, 1:4, 1:8, etc. The dilutions mentioned in this paper refer to the original dilution of the serum and not that which is attained after mixture with the complement and antigen. The complement was either fresh guinea pig serum titrated immediately before use or commercially available lyophilized guinea pig serum (Sharp and Dohme). 0.2 cc of the toxoplasma immune serum was mixed with 0.2 cc of complement (containing 2 units) and 0.2 cc of the toxoplasma antigen in one series and the normal rabbit brain antigen in a duplicate series. The tubes were incubated in a water bath at 37°C for 30 min, and 0.4 cc of a mixture consisting of equal parts of a 5% suspension of washed sheep erythrocytes and hemolysin (2 units per 0.2 cc) was added to each of the tubes which were then incubated at 37°C for another 30 min. The final reading was made after an overnight incubation in the refrigerator, the degree of fixation being expressed in the conventional manner, *i.e.*, complete fixation or lack of hemolysis as ++++ and no fixation or complete hemolysis as 0. Two plus fixation or better was regarded as significant. The usual anticomplementary and hemolysis controls were included in each test.

Complement Fixing Antibodies in Toxoplasma-infected Monkeys. The sera of normal rhesus monkeys tested fresh or within 24 hr after bleeding were either completely negative with both antigens or at best reacted only when used undiluted. After storage in the refrigerator, however, both normal

TABLE II.

The Appearance and Persistence of Complement Fixing Antibodies in the Serum of Monkeys with Experimental Toxoplasmosis.

Wks after inoculation	Monkey No. 281	392	393	408	409	410	411
Before inoculation	0 U*	0 (0)	0 (0)	*10 (10)*	*30 (20)*	*U (U)*	*Anti-complementary*
1	U (0)	16 (0)	0 (0)				
2	U (0)	16 (0)	U (0)	*20 (10)*	*40 (20)*	*30 (20)*	*60 (10)*
3	U (0)	8 (0)	0 (0)	*30 (10)*	*50 (20)*	*30 (10)*	*70 (10)*
4	2 (0)	8 (0)	8 (0)	*20 (5)*	*80 (40)*	*30 (10)*	*80 (10)*
5	4 (0)	8 (0)	8 (0)	*20 (5)*	*100 (70)*	*60 (5)*	*70 (10)*
6				20 (U)	*90 (50)*	50 (0)	60 (5)
7				10 (U)	90 (5)	10 (0)	30 (5)
8				10 (0)	90 (5)	10 (0)	60 (5)
9				0 (0)	80 (5)	0 (0)	
10				U (0)	90 (10)	10 (0)	60 (10)
12						U (0)	60 (10)
13				0 (0)	90 (0)	U (0)	
14		16 (U)	4 (U)		60 (5)		
17					30 (2)	U (0)	
28		2 (U)	4 (2)				
34						8 (2)	
45					4 (2)	U (U)	

Figures not in parentheses indicate titre with Toxoplasma-infected rabbit brain, while those in parentheses refer to the titre with normal rabbit brains.

The data in italics were obtained with sera which had been stored in the refrigerator for varying periods of time before the test, and show the degree of nonspecific fixation which develops in normal and immune monkey sera on storage (this does not occur when the sera are frozen). The tests on all the other sera were carried out within 24 hr after bleeding.

*U—undiluted.

and immune monkey sera have been found to develop the capacity to fix complement in varying degrees with both normal and toxoplasma rabbit brain antigens. With the exception of a certain number of sera (indicated in Table II) which were tested after various periods of storage in the refrigerator, all the other tests were, therefore, carried out within 24 hr after bleeding.

The sera of 7 rhesus monkeys which were infected by the intracutaneous and intra-abdominal routes and used in the neutralizing antibody investigation,[1] were studied at weekly intervals early after inoculation and at longer intervals subsequently. Monkeys 281, 392, 393, 408 and 409, received their toxoplasma in mouse brain suspension and monkeys 410 and 411 in rabbit brain suspension. Monkey 392, whose serum was entirely negative with both antigens before infection, developed specific complement fixing antibodies for the toxoplasma antigen as early as one week after inoculation, its serum giving a positive reaction in a dilution of 1:16. Definite evidence of specific complement-fixing antibodies appeared later in the other monkeys, the interval being as long as 4 weeks in some (Table II). While the maximum specific complement fixing titres varied from 1:4 in monkey 281 to 1:90 in monkey 409, it is not yet clear how much this variation depended on the individual monkeys and how much on the possible differing potencies of the antigens.

Unlike the neutralizing antibody the complement fixing antibody either completely disappeared (monkeys 408 and 410) or was diminished to very low titre after periods of 2 months or longer after infection (the antigen involved in some of these negative results gave high titres with other sera in simultaneous tests). It cannot be said, however, that persistent complement fixing antibody necessarily indicates a persistent infection since monkey 411 had a high titre of complement fixing antibody at a time when the toxoplasma had already disappeared from the body.[1]

Absence of Complement Fixing Antibodies in Toxoplasma-infected Rabbits, Dogs, and Cats. No complement fixing antibodies were found in the sera of 4 rabbits which were proved to be immune to toxoplasma. Four dogs, 2 adult and two 3 months old, and 3 cats were tested before and at intervals after experimental infection with toxoplasma, but no complement fixation was demonstrable.

Complement Fixing Antibodies in Human Beings. 63 specimens of serum from 56 individuals were tested for complement fixing antibodies. At no time did any of the human sera react with the normal rabbit brain antigen, while 10 individuals yielded sera which fixed complement to varying degrees with the toxoplasma rabbit brain antigen. These 10 positive reactions were associated with the following conditions: One infant (J.F.) who at one month of age presented hydrocephalus, cerebral calcification, and bilateral chorioretinitis and from whose cerebrospinal fluid Wolf, Cowen, and Paige isolated toxoplasma by animal inoculation, gave a positive reaction with only the undiluted serum at about 3 months of age and again at 4 months of age (we are indebted to Dr. Rustin McIntosh of New York, for these sera and the history); one 8-year-old boy (W.V.) exhibiting cerebral calcification and convulsions since 11 months of age had a serum with a titre of 1:4 (Dr. Bronson Crothers of Boston, kindly supplied this specimen and history); 3 mothers of infants in whom a diagnosis of congenital toxoplasmosis was considered on clinical grounds, and one mother who gave birth to an anencephalic monster, had titres varying from undiluted to 1:8; one was a man without any significant history other than rabbit hunting who was tested because his infant daughter to whom he gave blood for a transfusion was also positive. The sera of the 8 individuals just listed also had neutralizing antibodies against toxoplasma. The remaining two individuals (a patient and his mother) gave specific complement fixation reactions in repeated tests, but the neutralization tests with their sera were regularly negative. The patient was a 14-month-old infant who was suspected of having congenital toxoplasmosis by Drs. P. M. Levin and H. Moore of Dallas, Texas, since he presented microcephaly, cerebral calcification, chorioretinitis, convulsions and psychomotor

retardation. Two specimens of this infant's serum gave titres of 1:8, while two different specimens of the mother's serum had titres of 1:2. Among the sera of 43 selected individuals which were tested both for neutralizing and complement fixing antibodies, 8 had both, 20 had neutralizing but not complement fixing antibodies, 2 had complement fixing but not neutralizing antibodies, and 13 had neither antibody.

Discussion. Rhesus monkeys, but not cats, dogs, or rabbits, have been found to develop specific complement fixing antibodies during the course of experimental toxoplasmic infection. Unlike the neutralizing antibody, that responsible for complement fixation disappeared in many monkeys during convalescence. The complement fixing antibody, furthermore, differs from the neutralizing antibody in that it is destroyed neither by heating at 56°C for 30 min nor by storage in the refrigerator. Positive complement fixation reactions were also obtained with sera of human beings who were known or could be suspected of having experienced a toxoplasmic infection. The fact that as many as 20 individuals whose sera yielded positive neutralization tests gave negative complement fixation reactions may perhaps be dependent on the following factors: (1) the complement fixing antibody unlike the neutralizing antibody, did not persist for a very long time (as in most of the experimentally infected monkeys), (2) in none of these 20 individuals was the suspected toxoplasmic infection either active or recent, and (3) the toxoplasma antigen used in these tests may have been of low potency.

Further experiments are necessary to elucidate the conditions which will yield the most potent complement fixing antigen. We know of no reason, for example, why the extract of toxoplasma-infected rabbit brain should be an effective antigen, while similar extracts of mouse brain or other tissues containing even larger numbers of toxoplasma, should be devoid of any detectable complement fixing antigen. The only other investigation of this problem of which we are aware is that by Nicolau and Ravelo,[3] who reported positive complement fixation reactions with the sera and extracts of various tissues of experimentally infected rabbits (a smaller number of dogs, cats, guinea pigs, pigeons and monkeys were also tested) as the source of antibody and a methyl alcohol extract of powdered, dry, parasitized rabbit spleen as the antigen. This antigen gave no fixation in our hands and the sera of rabbits, dogs, and cats were found to be devoid of both complement fixing and neutralizing antibodies.

Summary. A frozen and thawed extract of toxoplasma-infected rabbit brain (but not similar extracts of certain other heavily parasitized tissues) yielded an antigen which fixed complement specifically in the presence of immune sera from experimentally infected rhesus monkeys but not rabbits, dogs, and cats. This complement fixing antibody appeared within 1 to 4 weeks after inoculation, but unlike the neutralizing antibody disappeared again in most monkeys, sometimes as early as 2 months. A certain number of human beings who were known or suspected to have experienced toxoplasmic infection also gave positive complement fixation reactions. However, twenty selected individuals whose sera had neutralizing antibodies against toxoplasma gave negative complement fixation reactions, but in none of these was there evidence of recent or active infection. While a positive complement fixation reaction does not in all cases indicate active or even recent infection, it is believed that this test may have its greatest usefulness in the rapid diagnosis of active toxoplasmosis.

[3] Nicolau, S., and Ravelo, A., *Bull. Soc. path. exot.*, 1937, **30**, 855.

13808

Effect of Certain Antiprotozoal Drugs on Toxoplasma *In Vitro* and *In Vivo*.*

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The recent demonstration that toxoplasma are pathogenic for human beings[1] stimulated the search for a chemotherapeutic agent that might be effective against this protozoon infection. Toxoplasma behave like obligate intracellular parasites with affinities for a great variety of fixed tissue cells and thus far it has not proved possible to cultivate them in media devoid of living, susceptible cells.[2] The effect on toxoplasma of certain compounds, known for their activity against various protozoa or other infectious agents, was, therefore, studied in two ways: (1) by incubating *in vitro* toxoplasma-containing exudate with the various drugs and then testing for survival of the toxoplasma by injecting the mixtures in mice, and (2) by administering the compounds under investigation to mice and rabbits infected with known amounts of toxoplasma.

The "R. H." strain of toxoplasma isolated in 1939 from a fatal case of human encephalitis[3] was used in these tests. This strain has been maintained in the laboratory by regular intracerebral passages in mice. Its virulence was such that 0.03 cc of a 1:10 suspension of infected brain inoculated intracerebrally killed all mice in 4 to 6 days. The minimal lethal dose (M.L.D.) was as a rule contained in the 10^{-4} to 10^{-5} dilution of infected brain, the mice inoculated with the smallest numbers of toxoplasma usually dying in 10 to 12 days. About 10 times as many toxoplasma were required to produce fatal infection by the intraperitoneal route, since the M.L.D. by this route was usually 0.3 cc of the 10^{-4} to 10^{-5} dilution. There was no evidence that this strain of toxoplasma gave rise to infection when less than lethal amounts were used in mice. Peritoneal exudate is abundant during the terminal stages of infection in mice inoculated with toxoplasma by the intraäbdominal route. This exudate diluted with heparinized (1:5,000) Tyrode's solution until there were 4 to 6 toxoplasma per high power field (×400), contained at least 10,000 M.L.D. per 0.3 cc administered by the intraäbdominal route, and was used in the *in vitro* tests.

In Vitro Tests. The procedure was as follows: 1.5 cc of diluted, heparinized, toxoplasma-containing, peritoneal exudate was mixed with 1.5 cc of various concentrations of drug in Tyrode's solution (pH 7.8) and incubated in a water bath at 37°C. One cc portions were removed at intervals of 3, 6, and 24 hr, and 0.3 cc was inoculated intraabdominally into each of 3 mice. For control, similar mixtures of toxoplasma and Tyrode's solution without drug were incubated and tested simultaneously at the same intervals. This procedure of testing the *in vitro* effect of various drugs is somewhat similar to that employed by Yorke and Murgatroyd[4] in their studies on trypanosomes.

The results of several such tests, summarized in Table I, indicate that quite a number of the compounds tested were able to destroy toxoplasma *in vitro*. This toxoplasmacidal effect was not altogether expected since, while most of the toxoplasma in the exudate were extracellular, many were intra-

* Presented in part at meeting of the Society of American Bacteriologists, Dec. 29, 1940. Abstract in *J. Bact.*, 1941, **41**, 80.

1 Sabin, A. B., Toxoplasmosis, a recently recognized disease of human beings, Advances in Pediatrics, Interscience Publishers, New York, 1942.

2 Sabin, A. B., and Olitsky, P. K., *Science*, 1937, **85**, 336.

3 Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

4 Yorke, W., and Murgatroyd, R., *Ann. Trop. Med.*, 1930, **24**, 449.

TABLE I.
Effect of Certain Compounds on Toxoplasma *in Vitro*.

Drug	Conc. of drug in mixture × 1000	Infectivity of mixture Toxoplasma and drug inoculated intra-abdominally into mice after incubation of mixtures at 37°C for 3 hr	6 hr	24 hr
Atabrin	1:10	0, 0, 0	0, 0, 0	0, 0, 0
,,	1:50	0, 0, 0	0, 0, 0	0, 0, 0
,,	1:100	7, 8, 9	10, 10, 11	0, 0, 0
Trypaflavin	1:10	9, 9, 9	0, 0, 0	0, 0, 0
Pot. Antimony Tartrate	1:10	11, 12, 12	0, 0, 0	0, 0, 0
Rivanol Lactate	1:10	10, 10, 12	0, 0, 0	0, 0, 0
Optochin	1:10	7, 9, 9	0, 0, 0	0, 0, 0
Mapharsen	1:20	7, 7, 7	0, 0, 0	0, 0, 0
Neosalvarsan	1:20	6, 6, 6	6, 7, 7	0, 0, 0
Tryparsamide	1:10	7, 7, 7	6, 6, 7	0, 0, 0
Quinine HCl	1:10	6, 6, 7	7, 8, 8	0, 0, 0
Bayer 205	1:10	7, 7, 8	7, 8, 9	12, 0, 0
Stibosan	1:10	6, 7, 7	7, 8, 8	13, 14, 14
4:4′ Diamidino Stilbene	1:10	7, 7, 7	8, 9, 9	12, 12, 12
Sulfanilamide	1:4	6, 6, 6	6, 6, 7	11, 11, 13
Na Sulfathiazole	1:4	6, 6, 6	6, 7, 7	10, 10, 10
Na Sulfapyridine	1:4	6, 6, 7	6, 6, 7	10, 10, 10
		6, 6, 8*	8, 8, 9	9, 9, 10
		6, 6, 9	7, 7, 7	8, 9, 9
Controls		6, 6, 6	6, 6, 6	9, 9, 10
Toxoplasma + Tyrode's solution		6, 6, 7	7, 7, 8	10, 11, 14
		7, 7, 7	6, 7, 7	9, 9, 10

*Numerals refer to day of death of inoculated mouse. 0 = mouse remained well.

cellular and presumably protected from the action of the drugs. The acridine derivatives, *atabrin, trypaflavin,* and *rivanol lactate* were all toxoplasmacidal, with atabrin being by far the most effective member of the group as well as the best of all compounds tested. In a concentration of 1:50,000 atabrin killed toxoplasma in 3 hr or less, while more than 6 and less than 24 hr were required for the same effect when the amount was reduced to 1:100,000. Among the antimony compounds *potassium antimony tartrate* in a concentration of 1:10,000 destroyed the toxoplasma in 3 to 6 hr while *stibosan* in the same concentration had no effect in 24 hr. Of the arsenic compounds tested *mapharsen* acted more rapidly than *neosalvarsan* or *tryparsamide.* In the quinine group, optochin was more effective than *quinine hydrochloride. Sulfanilamide, sodium sulfathiazole* and *sodium sulfapyridine* in concentrations of 1:4,000 (25 mg %), and *4:4′ diamidino-stilbene* in a concentration of 1:10,000 were completely without effect under the conditions of this test.

In Vivo Tests. Since the effect of the various drugs *in vitro* could be judged only by inoculating the toxoplasma-drug mixtures in animals, it was obviously impossible, solely on the basis of the preceding tests, to be certain that the action occurred either *in vivo* or *in vitro.* The following experiment illustrates, however, that even atabrin, which was the most potent toxoplasmacidal drug under the conditions of the so-called *in vitro* tests, was completely without effect *in vivo.* In the preceding tests atabrin, in a concentration of 2 mg % (*i.e.,* 1:50,000) was found to be capable of killing about 10,000 M.L.D. of toxoplasma contained in peritoneal exudate in the test tube in less than 3 hr. However, when the toxoplasma were injected intra-abdominally in mice weighing 14 to 16 g, and 2 mg of atabrin were injected intramuscularly simultaneously and daily thereafter, there was no apparent effect on the course of the infection even among the mice inoculated with as little as 1 to 10 M.L.D. (Table II). Twenty-one different compounds listed in

TABLE II.
Lack of Effect *in Vivo* of Drug (Atabrin) That Is Highly Toxoplasmacidal *in Vitro*.

Dilution of toxoplasma-infected mouse brain (0.3 cc intraäbdominally)	Untreated mice	Atabrin 2 mg intramuscularly daily beginning immediately after inoculation
10^{-1}	5, 5, 6*	5, 5, 5
10^{-2}	7, 7, 8	8, 8, 8
10^{-3}	9, 9, 10	9, 9, 10
10^{-4}	10, 10, 10	10, 10, 11

*Numerals refer to day of death of inoculated mouse.

TABLE III.
Compounds Without Effect on Toxoplasmic Infection in Mice.

Group	Compounds tested	Therapeutic effectiveness of group against other protozoa
Arsenic	Neosalvarsan Silver salvarsan Tryparsamide	Trypanosomes
Antimony	Potassium antimony tartrate	,,
	Fouadin	,,
	Stibosan	Leishmania
Bismuth	Bismuth subsalicylate	
Gold	Sodium aurothiomalate	
Iodine	Sodium iodide	Amoebæ
Quinine and related compounds	Quinine HCl Quinine SO_4 Optochin	Plasmodia
Acridine derivatives	Atabrin	,,
	Rivanol lactate	Trypanosomes
	Trypaflavine	
Aniline dyes	Methylene blue	,,
Urea derivative	Bayer 205 (Germanin)	,,
Aromatic diamidines	4:4′ Diamidino-stilbene	,,
	4:4′ Diamidino-diphenoxy pentane	Leishmania
	4:4′ Diamidino-diphenoxy propane	Plasmodia
	4:4′ Diamidino-diphenyl ether	Babesia

Table III were tested in this manner, most of them against about 1,000 M.L.D. of toxoplasma. Most of the compounds were administered in maximal dosage, *i.e.*, in amounts that were close to or within the range of toxicity; they were injected subcutaneously, intramuscularly, or intravenously daily beginning immediately after inoculation of the mice with the toxoplasma. Whenever there was any question regarding the cause of death, stained films of the lungs and viscera were examined for the presence of toxoplasma. The aromatic diamidines (kindly supplied by Dr. D. F. Robertson of Merck and Co.) were tested against 1 to 100 M.L.D. of toxoplasma because they were reported to exert a good therapeutic effect on leishmania and trypanosome infections (Lourie and Yorke).[5] None of the drugs listed in Table III had any effect on the course of toxoplasmic infection in mice. Prontosil administered intramuscularly daily in doses of 5 mg and sodium sulfapyridine twice daily in doses of 10 mg, subcutaneously at first and then intravenously, were also without therapeutic effect in mice.

Neosalvarsan, tryparsamide, stibosan, potassium antimony tartrate and acriflavine were also tested for possible prophylactic activity in mice. These drugs given 4 days, 2 days and immediately before infection with about 1,000 M.L.D. of toxoplasma, failed to modify the course of the experimental disease.

When toxoplasma are injected intracutaneously in rabbits there is an interval of

[5] Lourie, E. M., and Yorke, W., *Ann. Trop. Med.*, 1939, **33**, 289.

TABLE IV.

Effect of Several Drugs on Toxoplasmic Infection in Rabbits.

Rabbits received 0.3 cc of 10% suspension of toxoplasma-infected mouse brain intracutaneously, and all drugs were given intravenously immediately after injection and thereafter as indicated.

Drug	Dose and mode of administration	Result	
		Test 1	Test 2
Atabrin HCl	3 mg daily	10, 13*	—
Sodium sulfapyridine	50 " twice daily	10, 11	—
Neoarsphenamine	100 " daily	9, 10	
Silver arsphenamine	50 " "	S, S†	7, 7, 10, 10
Tryparsamide	100 " "	9, S	7, 7, 8
Untreated controls	—	11	8, 8, 9

*Numerals refer to day of death of each inoculated rabbit.

†S = rabbit survived for more than a month and exhibited neither a local skin lesion nor fever, and upon retest was found to be immune. The probability is that these rabbits were spontaneously immune at the time of the first test.

3 to 4 days before the local lesion develops and fever appears. There is then a phase of generalized infection with fever for 4 to 8 days, which almost invariably terminates in death with the strain of toxoplasma used in these studies. Atabrin, neoarsphenamine, silver arsphenamine, tryparsamide, and sodium sulfapyridine, administered as indicated in Table IV, were without effect on this type of toxoplasmic infection in rabbits.

Summary. In a search for a chemotherapeutic agent against toxoplasma a large number of drugs of known effectiveness against other protozoa were tested *in vitro* and *in vivo*. In the *in vitro* tests, heparinized peritoneal exudate, containing about 10,000 M.L.D. of toxoplasma, was mixed with certain concentrations of the compounds under investigation and after incubation at 37°C in a water bath for periods of 3, 6, and 24 hr, aliquot portions were injected intraäbdominally in mice to test for the survival of the toxoplasma. In this type of test atabrin in a concentration of 1:50,000 yielded a noninfective mixture in less than 3 hr; trypaflavin, rivanol lactate, potassium antimony tartrate, optochin (in concentrations of 1:10,000) and mapharsen (1:20,000) required more than 3 but less than 6 hr, and neosalvarsan (1:20,000), tryparsamide (1:10,000), and quinine HCl (1:10,000) required more than 6 but less than 24 hr to produce the same effect. Stibosan (1:10,000), 4:4′ diamidino stilbene (1:10,000), sulfanilamide (1:4,000), sodium sulfathiazole (1:4,000) and sodium sulfapyridine (1:4,000) were without effect on the toxoplasma even after 24 hr. All these and a number of other compounds had no therapeutic effect when they were administered parenterally to mice and rabbits immediately after injection of the toxoplasma and once or twice daily thereafter.

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Therapeutic Effectiveness of Certain Sulfonamides on Infection by an Intracellular Protozoon (Toxoplasma).*

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The fact that many drugs of known effectiveness against other protozoa were toxoplasmacidal *in vitro* without exhibiting any therapeutic action on toxoplasmic infection in mice and rabbits was reported in the preceding communication.[1] It was also pointed out that sulfanilamide, sulfathiazole, and sulfapyridine in concentrations of 25 mg % had no effect on toxoplasma *in vitro*, and that sodium sulfapyridine administered parenterally twice daily failed to modify the course of toxoplasmic infection in mice and rabbits.

In preliminary tests with the commonly used method of administering sulfonamides in the diet, it was found that mice receiving a diet containing 1% sulfathiazole resisted infection with many fatal doses of toxoplasma. A series of experiments designed to test the extent of the therapeutic or curative effect of certain sulfonamides on infection caused by this obligate intracellular protozoon parasite[2,3] were then carried out. The same virulent "R.H." strain of toxoplasma of human origin that was used in the preceding study was also used in the present experiments. The brains of mice succumbing after intracerebral inoculation were ground in a mortar without an abrasive and enough physiological salt solution or Tyrode's solution was added to make a 10% suspension. These suspensions were not centrifuged since toxoplasma are sedimented at relatively low speeds.

Comparative Effect of Sulfanilamide, Sulfapyridine, and Sulfathiazole. Mice weighing approximately 20 g were inoculated intraabdominally with 0.3 cc of various dilutions of toxoplasma-infected mouse brain suspension and divided into 4 groups. One group received a powdered diet without drug and the others were given the same diet containing 1% of sulfanilamide, sulfapyridine, or sulfathiazole. The mice were allowed to consume all they could eat. Random tests on the blood of these mice after they had been consuming these diets for several days indicated levels of 4 to 5 mg % in the sulfanilamide group, 2 to 3 mg % in the sulfapyridine group, and 1 to 2 mg % in the sulfathiazole group. The results shown in Table I indicate that sulfanilamide only prolonged the survival time and prevented death only in mice inoculated with about a single M.L.D. of toxoplasma; however, none of the mice receiving sulfapyridine or sulfathiazole succumbed while they were on the diet—not even those inoculated with 1,000 to 10,000 M.L.D. of toxoplasma. When these drugs were discontinued after 13 days, the mice inoculated with the larger numbers of toxoplasma succumbed, as a rule within 8 to 16 days after removal of the drug. That toxoplasma were the cause of death was established by demonstrating them in large numbers in stained films of the lungs. The mice were observed for 10 to 12 weeks before the experiment was discontinued. Surviving animals were found to be free of toxoplasma as demonstrated by subinoculation of their viscera into other mice.

Influence of Duration of Sulfathiazole Therapy on Extent of Curative Effect. It was apparent from the preceding experiment that sulfapyridine and sulfathiazole were capable of holding in check the proliferation of toxo-

* Presented in part at the 42nd annual meeting of the Society of American Bacteriologists, Dec. 29, 1940. Abstract in *J. Bact.*, 1941, **41**, 80.

1 Warren, J., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 15.

2 Sabin, A. B., and Olitsky, P. K., *Science*, 1937, **85**, 336.

3 Sabin, A. B., Toxoplasmosis, a recently recognized disease of human beings, *Advances in Pediatrics*, Interscience Publishers, Inc., New York, 1942.

TABLE I.

Comparative Effect of Sulfanilamide, Sulfapyridine, and Sulfathiazole in Diet on Toxoplasmic Infection in Mice.

Drug in diet	Duration of therapy, days	Dilution of toxoplasma-infected brain suspension (0.3 cc intraäbdominally) 10–1	10–2	10–3	10–4	10–5
None	—	5, 5, 6* 6, 6, 7	8, 8, 8 8, 8, 9	8, 9, 9 10, 10, 10	10, 11, 11	15, 0
Sulfanilamide 1%	34	6, 7, 7 9, 12, 12	12, 12, 15 16, 16, 21	17, 18, 20 21, 26, 0	28, 0, 0	—
Sulfapyridine 1%	13	*21, 21, 25* *25, 27, 29*	*25, 27, 43* *46*, 0, 0	0, 0, 0 0, 0	0, 0, 0	
Sulfathiazole 1%	13	K 22,† K 22† *26*	*25, 25*, 0	*27*, 0, 0	0, 0, 0	

*Numerals refer to day of death of inoculated mouse.

0 = mouse remained well.

Numerals in italics indicate mice which died of toxoplasmic infection after discontinuance of drug; numbers refer to days after inoculation.

†K 22 = mice were well but were killed on the 22nd day and toxoplasma were demonstrated in their viscera by subinoculation.

TABLE II.

Influence of Duration of Sulfathiazole Therapy on Extent of Curative Effect in Mice.

Exp.	Duration of therapy. 1% sulfathiazole in diet	Dilution of toxoplasma-infected brain suspension (0.3 cc intraäbdominally) 10–1	10–2	10–3	10–4	10–5
A	Controls—no drug in diet	—	5, 7, 7	8, 8, 9	9, 10, 10	14, 0, 0
	16 days	*29, 29, 31*	0, 0, 0	0, 0, 0	0, 0, 0	—
B	Controls—no drug in diet	5, 5, 5	7, 8, 8	8, 9, 9	10, 10, 11	0, 0, 0
		5, 6, 6	8, 8, 8	9, 9, 10	11, 11, 0	0, 0, 0
	30 days	*42, 42, 43*	*46*, 0, 0	0, 0, 0	0, 0, 0	*46*, 0, 0
		46, 47, 47	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	45 "	*62, 65, 66*	*107*, 0, 0	*88*, 0, 0	0, 0, 0	0, 0, 0
		68, 68, 81	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0

Legends as in Table I.

plasma. The question arose as to how long these drugs could hold back this proliferation and whether, if they were administered long enough, the body might finally rid itself of even the larger numbers of toxoplasma. Results of tests in which the 1% sulfathiazole diet was administered for periods of 16, 30, and 45 days to mice infected with various doses of toxoplasma, are shown in Table II. It may be seen that, although somewhat better results were obtained in these groups than in the one given the same diet for 13 days, there is, nevertheless, a limit to the number of toxoplasma, namely about 100 M.L.D., of which the mice can rid themselves, apparently regardless of the duration of therapy. It is of great interest that as long as the mice consume the drug, even those inoculated with the largest amounts remain well. Moreover, among the mice receiving the diet for 45 days a period of 20 to 60 days may elapse after removal of the drug before they succumb to toxoplasmic infection, although even the smallest numbers of toxoplasma require a maximum of only about 14 days to kill normal mice. The changes thus produced in the parasite or the host by the long administration of sulfathiazole would appear to be worthy of further investigation.

Tests on toxoplasma isolated from mice which ultimately succumbed after discontinuance of the drug revealed that they had not become drug-fast since infection produced by them in other mice was affected by sulfathiazole in the usual manner. The surviving mice from the groups treated with sulfathiazole for 30 and 45 days were used for two types of tests 4 months after they were in-

TABLE III.
Effect of Delaying Sulfathiazole Therapy on Toxoplasmic Infection in Mice.

Time after infection 1% sulfathiazole diet begun	Total time on diet, days	No. of mice	% of mice surviving one month	% of mice cured	Day of death of succumbing mice
Immediately	23	9	100	78	*37, 37*
2 days	28	9	67	56	8, 14, 20, *42*
4 "	26	19	47	0	8, 8, 8, 9, 11 12, 13, 13, 13, 21 *40, 40, 40, 42 42, 42, 42, 42, 44*
5 "	Duration of experiment	10	0	0	6, 6, 6, 8, 8 8, 8, 11, 11, 15
6 "	"	10	0	0	7, 7, 8, 8, 8 8, 8, 8, 8, 8
6 " 2 mg t.i.d. by stomach tube on 6th day + diet	"	10	10	0	7, 7, 7, 7, 7 7, 8, 8, 8, *46**
Untreated controls	—	20	0	0	7, 7, 7, 7, 8 8, 8, 8, 8, 8 8, 8, 8, 8, 8 8, 9, 9, 9, 10

Legends as in Table I.
*This mouse was on the sulfathiazole diet for 22 days.
All mice were inoculated intraäbdominally with 0.3 cc of a 1:100 suspension of toxoplasma-infected brain—about 100 M.L.D.

oculated. In one test, one mouse from each group inoculated with a given dilution of toxoplasma was sacrificed and a pool of its viscera was subinoculated in 3 other mice to establish the presence or absence of toxoplasma; 7 sulfathiazole-treated and 3 control mice were thus tested but no toxoplasma were found. The remaining mice were tested for immunity by an intraäbdominal injection of about 100 M.L.D. of toxoplasma, but none was found to be immune. Similar tests on other toxoplasma-infected, sulfathiazole-treated, surviving mice revealed that they were not resistant to reinfection and that immunity did not appear to be a factor in their survival.

Effect of Delaying Sulfathiazole Therapy. In the preceding experiments the mice were given the sulfathiazole diet immediately after inoculation with toxoplasma. It was of some interest to know how long it would be possible to withhold the drug and still obtain a therapeutic effect. Ninety mice were infected intraäbdominally with about 100 M.L.D. of toxoplasma; one group received no drug and others were given the 1% sulfathiazole diet at different times—immediately, 2 days, 4 days, 5 days, and 6 days after infection. Because the mice were already so sick on the 6th day that they consumed little or nothing of their diet, one group also received 2 mg of sulfathiazole by stomach tube 3 times during that day. The results shown in Table III indicate that when the drug was first given 2 days after infection, 56% of the mice were cured, *i.e.*, survived without relapse after discontinuance of the drug. This result was unexpected since it is known that toxoplasma undergo considerable proliferation during the first 2 days, and the preceding experiments indicated that infection with 1,000 M.L.D. or more could be held in check only while the drug was being administered and for a short time thereafter. When administration of the drug was delayed for 4 days, it was without effect in about 25% of the mice, prolonged the survival time in another 25%, and in the remainder the infection was checked while the drug was being consumed but all the mice died 10 to 14 days after therapy was discontinued. No therapeutic effect was obtained when sulfathiazole was first given 5 days after infection.

Effect of Sulfathiazole in the Diet on Infection by the Intracerebral Route. Severe

TABLE IV.
Effect of Sulfathiazole in Diet on Toxoplasmic Infection by Intracerebral Route in Mice.

Dil. of toxoplasma-infected brain suspension	Controls no drug in diet	1% sulfathiazole in diet for 21 days after intracerebral injection of toxoplasma	
		Mice dying while on drug	Mice dying after removing drug
10–1	4, 5, 5	0, 0, 0	0, 0, *33*
10–2	6, 6, 6	0, 0, 0	*36, 46, 51*
10–3	6, 6, 7	0, 0, 0	0, *54, 54*
10–4	7, 7, 7	0, 0, 0	0, 0, *46*
10–5	8, 9, 0	0, 0, 0	0, 0, 0

Legends as in Table I.

ependymitis, encephalitis and meningitis follow intracerebral injection of toxoplasma in mice. The results, shown in Table IV, indicate that sulfathiazole completely held in check this cerebral infection even when 10,000 M.L.D. were injected, as long as the drug was being consumed and for a considerable time thereafter. About 50% of the mice were cured, seemingly regardless of the size of inoculum, while the others died 12 to 33 days after discontinuance of the drug. When one considers the relatively short time in which the concentration of sulfathiazole in the blood reaches insignificant levels after discontinuance of the drug, and that the longest survival time after the minimal dose of toxoplasma was 9 days, it is rather remarkable that so many of the animals did not die for 25 to 33 days after the drug was removed from their diet.

Effect of Sulfathiazole on Toxoplasmic Infection in Rabbits. Rabbits were infected by the intracutaneous injection of toxoplasma-infected mouse brain suspension. The results shown in Table V indicate that 1% sulfathiazole in their diet had no effect on the infection. Examination of the blood of these rabbits revealed sulfathiazole levels which varied from 0 to 2 mg %, indicating that their irregular eating habits were probably responsible for the failure of the drug diet in the above experiment. In the previous communication it was reported that sodium sulfapyridine given intravenously twice a day was also ineffective. It remained to be determined, therefore, whether if proper levels of sulfonamide could be maintained, it would be possible to influence toxoplasmic infection in rabbits as well as in mice. Preliminary tests showed that after the intraäbdominal injection of 200 mg of sulfathiazole (suspended in 4 cc of H_2O) blood levels of 1 mg % or more were still present at 3 hr but irregularly or not at all later on. When sulfathiazole was administered intraäbdominally as described in Table V, it was possible completely to check the infection in the treated rabbits, even when the drug was first started 3 days after inoculation, at a time when a well-marked local lesion as well as high fever, indicative of widespread systemic involvement, were already present. This development of the local lesions and the fever before treatment was begun, affords good evidence that the rabbits were not spontaneously resistant. Small local lesions but no significant fever occurred in the rabbits treated immediately after infection; after discontinuance of the drug there were several rises of temperature which might have been on a toxoplasmic basis. The rabbits, however, survived and unlike the mice were resistant to reinfection.

Effect of Toxoplasmacidal Drugs in the Diet. Since in the unsuccessful therapeutic tests with the toxoplasmacidal drugs reported in the previous communication,[1] the drugs were all administered parenterally, it was desirable to repeat these tests with some of the same compounds incorporated in the diet. Groups of mice infected with 1, 10, 100, or 1,000 M.L.D. of toxoplasma by the intraabdominal route, were given diets containing 1% of either atabrin hydrochloride or quinine hydrochloride. However, no therapeutic effect of any kind was observed.

Discussion. The results presented in this communication are the first to demonstrate the inhibitory and, under certain conditions,

TABLE V.
Effect of Sulfathiazole Parenterally and in the Diet on Toxoplasmic Infection in Rabbits.

Exp.	Mode of administration of sulfathiazole	Time after infection drug started	Result
A	Controls—no drug	—	8,* 10, S
	1% in diet	Immediately	9, 9, S
		3 days (1st day of fever)	8, 9, 9
		5 days	6, 8, 12
B	Controls—no drug	—	8, 10, 11
	1.5-2 g per day intraäbdominally, divided doses every 3 hr between 6 A.M. and 6 P.M. and every 6 hr between 6 P.M. and 6 A.M.—for 12 days. Then 5% sulfathiazole in diet for 7 days	Immediately	S, S, S
	2 to 3 g per day intraäbdominally in divided doses as above—for 9 days. Then 5% sulfathiazole in diet for 7 days	3 days (1st day of fever)	S, S

*Numerals refer to day of death of each inoculated rabbit.
S = rabbit survived for 6 weeks or longer.
All rabbits infected by intracutaneous injection of 10% suspension of toxoplasma-infected mouse brain.

curative effect of certain sulfonamides on an infection by an apparently obligate intracellular protozoon parasite capable of multiplying in a great variety of fixed tissue cells. The behavior of sulfathiazole and sulfapyridine in this infection, particularly their capacity to hold in check the proliferation of large numbers of toxoplasma as long as the animal consumes the drug and, under certain conditions, also for long periods thereafter, appears to be different from the effects of these drugs observed in bacterial infection and from the reported action of sulfanilamide on *Plasmodium knowlesi* in monkeys.[4] Sulfanilamide killed these plasmodia *in vitro* and several daily injections, or as little as one dose of 1 g of sulfanilamide by mouth, sufficed to eradicate completely both acute and chronic infections in monkeys.

Summary. Sulfathiazole and sulfapyridine, although without effect on toxoplasma *in vitro,* have been found to have a complete inhibitory and under certain conditions, curative action on toxoplasmic infection in mice when administered in 1% concentration in the diet. Sulfanilamide did not have a similar effect. Mice infected with 100 M.L.D. or less were cured when 1% sulfathiazole was given for 16 days or longer. Although mice infected intraäbdominally with a single M.L.D. had a maximum survival time of about 14 days, animals infected with 1,000 to 10,000 M.L.D. remained well as long as they were consuming the drug diet and for variable periods thereafter, but ultimately succumbed to toxoplasmic infection. It seemed remarkable that many such animals which had received the 1% sulfathiazole diet for 45 days did not succumb for 1 to 2 months after discontinuance of the drug. Sulfathiazole in the diet was equally effective when the toxoplasma were injected intraäbdominally or intracerebrally. Apparently because of the irregular eating habits of rabbits, 1% sulfathiazole in the diet was without effect on toxoplasmic infection in them. However, when large doses of sulfathiazole were administered parenterally at regular intervals, day and night, a distinct curative effect was obtained even when the drug was first administered 3 days after infection by the intracutaneous route, at a time when well developed local lesions and fever, indicative of a generalized systemic infection, were present. Atabrin and quinine hydrochloride which were previously found to be toxoplasmacidal in *in vitro* tests, were without effect on toxoplasmic infection in mice when administered in the diet.

4 Coggeshall, L. T., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 768; *Am. J. Trop. Med.*, 1938, **18**, 715; *J. Exp. Med.*, 1940, **71**, 13.

13810

Some Biologic and Immunologic Characteristics of a Pleuropneumonia-Like Microörganism of Human Origin.

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The first unequivocal isolation from human beings of microörganisms morphologically and culturally similar to those of bovine pleuropneumonia was reported by Dienes in 1940.[1] Using an ascitic fluid enriched agar medium he was frequently able to obtain such microorganisms from the secretions of the uterine cervix of female patients. This work was subsequently corroborated by Smith,[2] who in addition succeeded in cultivating a similar strain from the urethral discharge of a man with urethritis and arthritis in whom gonococci were not recovered.

Dienes and Smith[3] have recently reported the isolation of pleuropneumonia-like microorganisms from the genital tracts of about 22% of 129 unselected male and female patients, for the most part from the secretions of the cervix uteri. They conclude that these organisms may dwell in the female genital tract without causing any obvious pathologic changes. However, striking evidence is also presented which suggests that they may also be the incitants, either alone or in combination with other bacteria, of a clinical picture that is similar to gonococcal infection in the female. In males they have thus far only been isolated from cases of chronic prostatitis, and mainly in patients with a history of previous gonococcal infection. Two instances are cited in which it appears that the microorganisms were transmitted from husband to wife by sexual intercourse.

The very scanty growth of these strains in fluid media was a barrier to a proper study of the biologic, pathogenic and serologic characteristics of this new group of microorganisms. Dr. Louis Dienes very kindly gave us one of his strains in order that we might attempt to obtain a satisfactory growth in broth. This strain, No. 4330, was originally isolated from the cervix uteri and was received by us in the form of scrapings from ascitic fluid agar cultures.

The medium we have employed consisted of an infusion broth, (prepared from fresh beef hearts), containing 1% of Pfanstiehl peptone and 0.5% glucose. The reaction of this medium after sterilization and before the addition of serum was pH = 8.0. This basic medium was enriched by the addition of either filtered ascitic fluid to a concentration of 30% or of rabbit serum to a concentration of 10%. The final reaction of the rabbit serum broth was about pH = 8.2. Transfers were made by pipette using 0.1 cc of culture into about 5.0 cc of the medium.

The original agar scrapings containing this strain were inoculated on the surface of ascitic agar plates where an abundant growth appeared within 48 hr. Blocks of agar containing these colonies were then removed from these plates and dropped into tubes of 30% ascitic fluid broth and into 10% bovine serum broth. No growth was observed in the latter medium and this series was discontinued. However, a faint, diffuse opalescence appeared in the ascitic broth after 5 days. This same culture was "passaged" blindly on the fourth day, *i.e.*, before any gross evidence of growth was apparent and a similar faint "growth" appeared in this second broth transfer at the end of 4 days. That this opalescence actually represented multiplication of the microorganisms was proven by transferring some of the fluid culture onto the surface of 30% ascitic fluid agar plates where numerous colonies appeared in 72 hr. After 6 passages in ascitic fluid broth it was noted that the

1 Dienes, L., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 468.

2 Smith, W. E., *J. Bact.*, 1942, **43**, 83 (Abstract).

3 Dienes, L., and Smith, W. E., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 99.

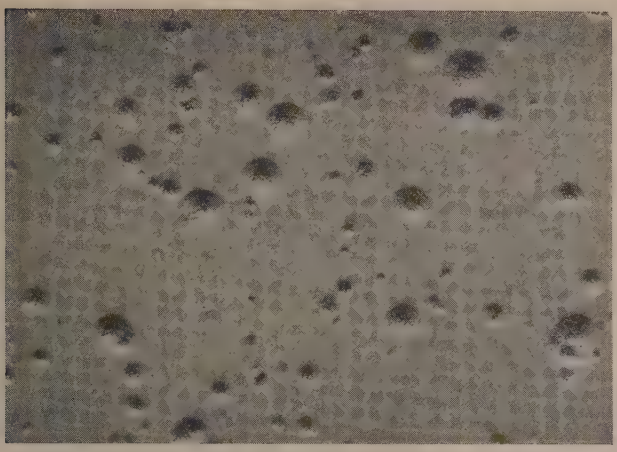

FIG. 1.
Microörganism of pleuropneumonia group of human origin, strain No. 4330 (Dienes). 72-hr culture; colonies on nutrient agar containing 30% ascitic fluid. Oblique lighting. × 95.

growth was heavier and was regularly noticeable within 72 hr. The eighth passage was transferred to 10% rabbit serum broth. Adaptation to this medium was rapid and after the first 4 passages growth regularly appeared in 48 hr. At best it was never very heavy although when 0.1 cc of such a broth culture was plated on the surface of enriched agar innumerable colonies developed. (Fig. 1).

Broth cultures retained their viability in the refrigerator for at least 2 weeks. Unlike certain of the rodent types of organisms of this group, this human strain remained viable when kept in the glucose serum broth at 37°C for as long as 7 days.

The human strain was tested for pathogenicity in mice using 24 hr (17th passage), 48 hr (15th passage), and 72 hr (6th passage) rabbit serum broth cultures. One-half cc was inoculated intravenously into groups of 4-week-old mice. The numbers of mice were 5, 8, and 10 for the 3 ages of culture respectively. All animals were observed daily for one month during which period they remained well and no gross evidence of arthritis was apparent.

When this human strain of the pleuropneumonia group was well adapted to the rabbit serum fluid medium, serological studies were carried out.

Antiserum was prepared in rabbits by the repeated intravenous injection of the resuspended sediments of broth cultures. The cultures used as sources of antigen had been passaged in rabbit serum broth for 34 generations. One hundred cc of a 10% rabbit serum broth culture, 4 days old, was spun on the angle centrifuge at 3000 rpm for one hr. The supernatant fluid was discarded and the sediment, resuspended in about 5.0 cc of physiological saline, was inoculated. The schedule of immunization consisted of 4 injections of 2.5 cc of the suspension of sedimented culture intravenously every other day, and 4.5 cc for the last injection. Blood was obtained from the heart 3 weeks after the last injection. Two rabbits were thus immunized. One animal was found dead 24 hr after the last injection. Necropsy revealed no gross changes. The second animal remained well throughout the course of inoculations.

Antigens for agglutination were prepared in the same manner as for injection except that the sediments were resuspended in 0.45% saline which appears to yield a smoother suspension.

TABLE I.
Reaction of an Antiserum to the Human Strain No. 4330 with the Homologous Antigen and Types A, B, and C of the Mouse Pleuropneumonia Group.

Final dilution of serum for human strain No. 4330	Antigen			
	Human No. 4330	Mouse		
		Type A	Type B	Type C
1:2	+++	0	0	0
1:10	++++	0	0	0
1:100	++++	0	0	0
1:500	++++	0	0	0
1:1000	++++	0	0	0
1:2000	0	—	—	—

The immune serum prepared against strain No. 4330 agglutinated this organism to a final serum dilution of 1:1000. However, this serum did not react to any degree with types A, B, and C microörganisms of the mouse pleuropneumonia group (Table I) (*Cf.* Sabin[4]). The type A, B, and C antigens were prepared in the same manner as the human strain using 48 hr 10% rabbit serum broth cultures. Conversely, antisera against types A, B, C, D, E of the mouse pleuropneumonia group and L3, and L4 of rats, did not agglutinate suspensions of the human strain. Thus, no serologic relation was found between the pleuropneumonia-like microörganism of human origin and representative types from the mouse and rat.

Summary. By means of serial passage in 10% rabbit serum broth it has been possible to secure sufficient growth in fluid media of a strain of pleuropneumonia-like microorganism derived from the human cervix uteri to permit serologic and pathogenic studies.

This strain was found not to be pathogenic for mice when cultures were injected intravenously, and no evidence of a toxin was observed.

Immune serum was prepared by the repeated inoculation of rabbits with saline-resuspended sediments of rabbit serum broth cultures. A satisfactory agglutinating antigen was prepared in the same manner. This serum agglutinated the homologous human antigen to a dilution of 1:1000, but failed to agglutinate antigens prepared similarly from Types A, B, and C of the mouse pleuropneumonia group.

Antisera to Types A, B, C, D, E from mice and L3 and L4 from rats, even when used undiluted, failed to agglutinate the human strain. From what is known of the pleuropneumonia group, microörganisms native to one host are rarely pathogenic for another and usually are serologically distinct. With a strain that is native to man one would expect neither significant serological cross-reactions with rodent strains nor would one expect pathogenicity in rodents. As a matter of fact it may not be possible to establish whether or not the human strains are pathogens or saprophytes by animal inoculation. The development of antibodies in human beings carrying such strains and tests in man may be necessary to elucidate this question.

4 Sabin, A. B., *Bact. Rev.*, 1941, **5**, 1.

13811

Inability of Pimelic Acid to Replace Biotin as a Growth Factor for *Lactobacillus casei.*

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Pimelic acid was first encountered in biological material when Mueller[1] isolated it from urine and demonstrated that it possessed growth factor properties for the diphtheria bacillus. Pimelic acid has since been shown to stimulate the growth of *Colpidium campylum.*[2] In a study of the growth factor requirements of the diphtheria organism, du Vigneaud *et al.*[3] found that pimelic acid could be replaced by biotin. From a consideration of the biotin structure they were led to postulate that in the absence of biotin the diphtheria organism is able to utilize pimelic acid in lieu of biotin for purposes of growth.

Since at present microbiological methods of assay must be resorted to for the determination of biotin, the question of the specificity of the response to biotin by other microörganisms arose. In this investigation the extent to which pimelic acid may replace biotin for growth and acid production by *Lactobacillus casei* has been studied. The requirement for biotin by this organism forms the basis for the assay procedures of Shull, Hutchings, and Peterson[4] and of Landy and Dicken.[5] Yeast has already been shown to be incapable of utilizing pimelic acid as a growth factor in place of biotin.[3]

In determining the ability of pimelic acid to replace biotin for *L. casei* the assay technique and procedure of Landy and Dicken[5] has been employed. Folic acid was supplied in the form of a concentrate through the courtesy of Dr. E. L. R. Stokstad. The pimelic acid used (mp 102-103°C) was obtained from Eastman Kodak. Pimelic acid was tested both in the presence and absence of added urea since it was considered possible that biotin might arise from a condensation of pimelic acid, cystine, and urea. The medium of Landy and Dicken contains 1 mg of cystine per culture tube. Table I presents the results of several experiments in which the activities of pimelic acid and biotin have been compared.

TABLE I.
Inability of Pimelic Acid to Replace Biotin as a Growth Factor for *Lactobacillus casei.*

Test material per culture tube	Experiment No. 1 Production of acid ml	Experiment No. 2 Production of 0.1 N acid ml	Experiment No. 3* Production of 0.1 N acid ml
None	2.60	3.08	3.00
.0005 γ biotin	9.30	8.00	7.55
.0010 "	11.60	10.30	10.10
.0025 "	14.60	12.60	12.55
.0005 γ pimelic acid	3.00	—	—
.001 " "	2.85	—	—
.01 " "	2.65	—	—
.05 " "	—	3.00	3.00
.10 " "	3.30	3.07	2.98
.20 " "	—	3.00	3.00
.50 " "	—	3.00	3.08
1.00 " "	—	3.00	3.00
2.00 " "	—	2.50	3.06
3.00 " "	—	2.95	3.00
5.00 " "	—	3.00	2.98

*Tubes in this series contained 1 mg per tube added urea in addition to the regular constituents of the test medium.

Summary. Pimelic acid was found to be ineffective in lieu of biotin as a growth factor for *L. casei.*

1 Mueller, J. H., *J. Bact.*, 1937, **34**, 163.

2 Hall, R. P., *Arch. Protistenk.*, 1939, **92**, 315.

3 du Vigneaud, V., Dittmer, K., Hague, E., and Long, B., *Science*, 1942, **96**, 186.

4 Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

5 Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

13812

Prevention by Succinyl Sulfathiazole of Ulcerative Cecitis in Rats.

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In a previous note[1] we reported that inclusion of sulfaguanidine in the normal ration prevented the development of ulcerative cecitis in a rat colony in which the disease was occurring in most of the untreated animals. It seemed of interest, therefore, to study the prophylactic value of the new drug succinyl sulfathiazole* which has already been shown to be highly effective in eliminating various organisms from the intestinal tract. Succinyl sulfathiazole acts, as Poth[2] has shown, by breakdown in the bowel with liberation of sulfathiazole which is the effective agent.

Ulcerative cecitis of rats has been well described recently by various observers and in order to save repetition the reader is referred to these papers.[3] Suffice it to say that the main features are superficial ulceration of the mucosa of the cecum associated with inflammatory thickening of the bowel wall, marked pericecitis and regional adenitis. There is considerable superficial resemblance to so-called regional enteritis in man. Although paratyphoid bacilli are intimately associated with rat cecitis we have shown[4] that these organisms are probably not the primary cause (which at present remains unidentified) although there can be little doubt that the disease is an infection.

Experiments. Twelve normal male rats, 64 days old, were placed on the stock diet of the laboratory† in which 1% of succinyl sulfathiazole was incorporated. As each rat eats 10 to 15 g of the ration daily he ingests 0.1 g of the drug, which would correspond in a 60 kg man with a daily dose of 30 g. Eight litter mate rats were placed on the stock diet without the drug as controls. The animals all throve and there was no essential difference in the weight of the two groups. After 110 days on the drug (174 days old) the rats were exsanguinated under ether anesthesia.

All the animals looked well, and no lesions of any sort were found in the rats which had received succinyl sulfathiazole. The cecums were large and contained bulky, soft masses of feces, slightly lighter in color and with less odor than in the controls. No ulceration was found in the cecum and there was no enlargement of regional lymph nodes. Among the untreated litter mate controls, on the other hand, 38% showed typical lesions of ulcerative cecitis.

In addition to this group of litter mate

TABLE I.
Incidence of Ulcerative Cecitis in Rats Receiving Succinyl Sulfathiazole and in Controls.

	No.	Cecitis present	% showing cecitis
Rats receiving Succinyl sulfathiazole	12	0	0
Untreated litter mate controls	8	3	38
Other control groups of same age	18	8	
	13	7	
	12	7	
	12	4	48
	8	5	
	8	4	
	8	3	
	6	3	
	85	41	

† Casein 10, corn meal 73, linseed cake meal 10, sardine oil 3, alfalfa 2, bone ash 1.5, and NaCl 0.5 parts.

1 Bloomfield, A. L., and Lew, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 363.

* We are indebted to Sharp and Dohme, Inc., for the succinyl sulfathiazole used in these experiments.

2 Poth, E. J., Knotts, F. L., Lee, J. T., and Innui, F., *Arch. Surg.*, 1942, **44**, 187.

3 Stewart, H. L., and Jones, B. F., *Arch. Path.*, 1941, **31**, 37.

4 Bloomfield, A. L., and Lew, W., to be published.

controls we have records of the incidence of cecitis in other groups of normal rats of approximately the same size raised on the stock diet. These findings are summarized in Table I.

Pooled serum from the rats receiving succinyl sulfathiazole was tested for its sulfonamide content which was 0.8 mg %.

Conclusion. Succinyl sulfathiazole incorporated in the stock ration completely prevented the development of ulcerative cecitis in rats.

13813

No Demonstrable Substance Causing Increased Capillary Permeability in Lymph from an Injured Area.

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The possibility that the hypothetical "toxic" factor responsible for traumatic shock was present in lymph was considered by Dragstedt and Mead.[1] Since they found that lymph obtained from the thoracic duct of a dog after intestinal trauma did not cause a fall in blood pressure when injected intravenously into a test animal, they concluded that no "toxic" substance was present in this lymph. It seemed possible, however, by using increased capillary permeability as a criterion of shock rather than depression of blood pressure, that the presence of a "toxic" substance in the lymph might be demonstrable.

Technic. Large, mongrel dogs were used. Under spinal anesthesia, a cannula was tied into a collecting lymphatic trunk between the femoral artery and vein. Lymph was collected by gentle intermittent pressure upon the tissues of the extremity and was stored in chilled tubes. After several control samples of lymph had been obtained, the extremity was injured either by heat or by trauma. An immediate increase in the flow of lymph was observed. Within 5 min the lymph became turbid and soon thereafter became blood tinged. Samples of lymph were collected at intervals for 3 hr. A tourniquet was then tightly applied about the traumatized extremity and 3 mg per kg of the blue dye T-1824 were injected intravenously.

Two-tenths of a cm^3 of each lymph sample was injected intracutaneously into the abdominal wall of the dog from which the lymph had been obtained. An equal amount of each sample was injected into another dog which had previously received the same amount per kg of the blue dye. At the end of 15 min the degree of staining of the area where the lymph had been injected was noted. When the lymph contained a factor

TABLE I.

Changes in Capillary Permeability Indicated by Diffusion of Blue Dye into Area Injected with Lymph Obtained from Femoral Region Before and After Trauma.

Time lymph sample obtained	Dog A "Donor"	Dog B "Recipient"
10:45	0	+
10:53	0	0
11:00	0	0
11:12	0	0
Trauma to leg		
11:18	0	++
11:37	0	+
11:50	0	0
12:50	0	++
2:00	0	++++
2:27	0	+++
Donor Serum		
Arterial	0	+++
Venous	0	+++
Recipient Serum		
Arterial	++++	0
Venous	++++	0

[1] Dragstedt, C. A., and Mead, F. B., *J. Am. Med. Assn.*, 1937, **108**, 95.

TABLE II.

Changes in Capillary Permeability Indicated by Diffusion of Dye into Area Injected with Arterial Serum, Arterial Plasma, Venous Serum and Venous Plasma Obtained from Each of the Dogs.

	Dog No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Dog No. 1						
Arterial Serum	0	++++	++++	++++	+	++++
Venous "	0	++++	++++	++++	+	++++
Arterial Plasma	0	++++	++++	++	+	0
Venous "	0	++++	++++	++	++	0
Dog No. 2						
Arterial Serum	++++	0	++++	+++	++	++++
Venous "	++++	0	++++	+++		++++
Arterial Plasma	++++	0	++++	+++	+	++++
Venous "	++++	0	++++	+++	+	++++
Dog No. 3						
Arterial Serum	++++	++++	0	++	+	++++
Venous "	++++	++++	0	++	+	++++
Arterial Plasma	++++	++++	0	++	+	+++
Venous "	++++	++++	0	++	+	+++
Dog No. 4						
Arterial Serum	++++	++++	++++	0	++	++++
Venous "	++++	++++	++++	0	++	++++
Arterial Plasma	++++	++++	++++	0	++	+
Venous "	++++	++++	++++	0	++	0
Dog No. 5						
Arterial Serum	++++	++++	++++	++	0	++++
Venous "	++++	++++	++++	++	0	++++
Arterial Plasma	++++	++++	++++	++	0	+
Venous "	++++	++++	++++	++	0	0
Dog No. 6						
Arterial Serum	++++	++++	++++	+++	+	0
Venous "	++++	++++	++++	+++	+	0
Arterial Plasma	++++	++++	++++	++	+	0
Venous "	++++	++++	++++	++	+	0

which caused an increase in capillary permeability, dyed plasma leaked out of the blood vessels and stained the site of injection.

Results. Lymph obtained from a dog's extremity before trauma produced no increase in capillary permeability when injected intracutaneously into another dog. After trauma, the lymph contained some factor which brought about increased capillary permeability as shown in Table I. However, serum from the arterial and venous blood also contained a permeability factor. When the lymph obtained from the dog after trauma was injected into the skin of his own abdominal wall, however, no increase in permeability could be shown. When serum from the arterial and venous blood of the recipient was used in the donor, a positive reaction was obtained.

These findings suggested the possibility that dogs might show a reaction to the serum of other dogs when injected intracutaneously and yet not to their own serum. Arterial and venous blood was drawn from each of 6 dogs selected at random. Serum and plasma were obtained from each sample. Two-tenths of a cm^3 of each specimen was injected intracutaneously into the skin of the abdominal wall of each dog. As can be seen from Table II none of the dogs reacted locally to its own arterial or venous plasma or serum, but they all reacted to injection of material from other dogs. Fig. 1 shows the reaction observed in dog 3. There were variations in the degree of reaction in the different dogs.

Discussion. It has been clearly shown by Field and Drinker[2] that lymph obtained under resting conditions contains only a small amount of protein but that the concentration of protein rises rapidly after injury to the region from which the lymph is flowing. It seems probable that the factor in the lymph of the donor responsible for the local increase in capillary permeability in the recipient was the same as that present in the circulating blood of the donor.

[2] Field, M. E., and Drinker, C. K., *Am. J. Physiol.*, 1931, **98**, 378.

FIG. 1.

Increased capillary permeability shown by diffusion of dye into area injected with homologous arterial and venous serum and plasma. No reaction was obtained from dog's own serum or plasma as shown by absence of staining in lower 4 squares, Dog No. 3, Table II.

Menkin[3] has stated that the substance found in exudates which is responsible for producing an increase in capillary permeability is also present in blood, "although biological tests indicate that it is present there in definitely smaller quantities than in an inflammatory exudate." We have not been able to demonstrate that lymph coming from a traumatized area contains a substance any more capable of producing an increase in capillary permeability than normal plasma.

Conclusions. 1. Lymph obtained from a traumatized extremity contained a substance which produced an increase in capillary permeability when injected into another dog. 2. When tested upon the dog from which the lymph was obtained, there was evidence that a toxic substance, capable of causing an increase in capillary permeability, appeared after mechanical or thermal trauma. 3. Arterial and venous serum and plasma contained a factor which increased the capillary permeability when injected intracutaneously into other dogs but not when injected into the dog from which the blood had been obtained. 4. It is likely that the presence in the lymph of a substance capable of producing an increase in capillary permeability is dependent upon the appearance, after trauma, of blood plasma in the lymph draining from the extremity.

[3] Menkin, V., *J. Exp. Med.*, 1936, **64**, 485.

13814

Preparation of Purified Influenza Virus.*

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Considerable effort has been directed, in recent years, toward the separation of viruses from the tissues within which they are propagated. This has met with variable degrees of success, and virus preparations ranging from complete to questionable states of purity have been reported by numerous investigators.

In the course of a study on active immunization against epidemic influenza conducted in this laboratory,[1,2,3] the obvious advantages of purified antigens for immunologic and serologic application prompted the investigation of methods for the purification of influenza virus.

Preliminary experiments indicated that influenza virus is in some respects rather labile and is readily affected by certain types of treatment which leave many other viruses relatively unimpaired. Attempts at salting out, extraction with ether or alcohol, mild hydrolysis, the addition of oxidizing or reducing substances and wide changes in pH, all reduced the activity of the virus quite drastically. Similar observations on some of these variables, studied in greater detail, were reported by Stock and Francis.[4] It became apparent, therefore, that other methods, less deleterious to the infectivity of the virus, would be required.

A technique of ultrafiltration, perfected by Bronfenbrenner and employed by him for the filtration of lytic filtrates of bacteriophage, was selected as suitable for this investigation. The details of the technique are given in the original report by Bronfenbrenner.[5]

The virus used for purification was obtained from 20% stock suspensions of influenza A virus prepared from pools of several hundred lungs of infected mice sacrificed 3-4 days after inoculation. The material was stored in a dry-ice cabinet at about —70°C, from which quantities were removed and thawed as required. These suspensions were diluted to 1% with saline or Tyrode's solution and subjected to a preliminary filtration through a Berkefeld V candle. Because of the variability of the results obtained with Berkefeld filters, due to frequent clogging, slow filtration and the occasional excessive loss of virus, filtration through paper pulp in a Buchner funnel was later substituted. There resulted a more rapid removal of the coarse particles, the loss of virus being on the average less than that following Berkefeld filtration.

The crude filtrate was then passed through the ultrafiltration apparatus in which an alundum thimble served as a support for a collodion membrane. Trials with collodion of various concentrations indicated that 2% collodion-acetate membranes were most consistently suitable for selective fractionation of the 1% virus filtrate. Membranes of this density, prepared within alundum thimbles, retained all of the virus while the greater proportion of inactive associated materials filtered through. However, the ease with which the separation was accomplished depended largely upon the degree of preliminary clearing.

Following ultrafiltration, the virus-containing residue was washed by permitting 200-400 cc of distilled water to pass through the

* Aided by a grant from the Metropolitan Life Insurance Company on the recommendation of the Influenza-Pneumonia Commission.

1 Siegel, M., and Muckenfuss, R. S., *Am. J. Hyg.*, 1941, **34** (Sect. A), 39.

2 Siegel, M., Muckenfuss, A. S., Schaeffer, M., Wilcox, H. L., and Leider, A. G., *Ibid.*, 1942, **35**, 55.

3 Siegel, M., Muckenfuss, R. S., Schaeffer, M., Wilcox, H. L., and Leider, A. G., *Ibid.*, 1942, **35**, 186.

4 Stock, C. C., and Francis, T., Jr., *J. Exp. Med.*, 1940, **71**, 661.

5 Bronfenbrenner, J. J., *Ibid.*, 1927, **45**, 873.

filter, resulting in an additional removal of inactive, diffusible substances. The residue was then recovered by removing the alundum thimble from the apparatus, adding small quantities of Tyrode's solution and dislodging the adherent material from the surface of the collodion membrane with the aid of a sterile swab.

Correcting for comparable volumes, assays were made of the relative infectivity and nitrogen content of the original 1% suspension, crude filtrate, wash water and concentrated residue. Titrations for infectivity were made in tenfold dilutions, each inoculated intranasally into 4 young Swiss mice under ether anesthesia, and the end-points determined by the 50% mortality method.[6] Nitrogen determinations were made by the micro-Kjeldahl method.

The results obtained in 10 experiments yielding substantially similar data are exemplified by Table I.

TABLE I.

Exp. No.	Material tested	Infectivity (Final dilution)	Nitrogen content (mg/cc)
1	1% crude suspension	$10^{-6.0}$	.318
	Berkefeld filtrate	$10^{-4.5}$	.277
	Ultrafiltrate	0	.182
	Wash water	0	.036
	Virus residue	$10^{-4.5}$	.014
2	1% crude suspension	$10^{-6.0}$	.377
	Paper pulp filtrate	$10^{-5.0}$	.229
	Ultrafiltrate	0	.091
	Wash water	0	.040
	Virus residue	$10^{-4.5}$	.021

From the data presented above it will be seen that while there was some reduction in nitrogen as a result of the preliminary crude filtration, there was also a considerable diminution in virus activity. From this point on, however, the bulk of the nitrogenous material was removed by ultrafiltration with relatively little or no loss in virus. Washing with water further reduced the nitrogen content so that the final residual virus recovered was depleted of 90-95% of the inactive and presumably non-essential associated nitrogen.

[6] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

If a comparison of the infective titers and nitrogen contents were to be made between the crude suspensions and final virus residues, it might be concluded that essentially no purification was effected but that the result could easily be duplicated by mere dilution. But since, strictly speaking, purification begins with ultrafiltration, it will be observed that using the crude filtrates as the starting point, considerable amounts of nitrogen were removed with little or no loss of virus.

At about the time that these data were obtained, Salk reported the partial purification of influenza virus by adsorption on calcium phosphate.[7] Following the same technique results comparable to those described by Salk were obtained. However, if instead of the final dialysis, as prescribed by Salk, the material was subjected to the ultrafiltration and washing described above, it was possible to obtain a product containing only about 1.5% of the original nitrogen; but a slight depletion in the infectivity of virus occurred during this procedure. The results are illustrated in Table II.

TABLE II.

Material	Infectivity (Final dilution)	Nitrogen content (mg/cc)
10% crude virus susp.	$10^{-6.0}$	3.520
Virus susp. after adsorption on calcium phosphate and resusp. in sodium citrate and hydrochloric acid	$10^{-6.0}$	0.273
Adsorbed and resuspended virus after ultrafiltration, washing and final resuspension in Tyrode's solution	$10^{-5.5}$	0.048

Besides yielding a more purified product, the combination of these two procedures with the substitution of ultrafiltration for dialysis has the added advantage of permitting the washing of the residue with large amounts of water and subsequent concentration to any desired volume with relative rapidity.

Tests of the comparative immunizing properties of the original virus suspensions and purified products have shown that no ap-

[7] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 709.

parent impairment of antigenicity occurs as a result of the purification. The solid protection against 1000 minimal infective doses induced in groups of mice inoculated intraperitoneally with 0.5 cc of the crude virus suspension, was produced as well in others with 0.5 cc of the purified preparations restored to their original volumes.

That a further step may be available for the production of more completely purified virus is indicated by some preliminary experiments entailing the use of proteolytic enzymes for the splitting off of associated protein material. While the details have not been worked out thoroughly, evidence was obtained that the infectivity of influenza virus is not affected by the separate action of trypsin, chymotrypsin and ribonuclease on suspensions of infected mouse lungs. When 1.8 cc amounts of a 10^{-3} dilution of mouse lung suspension were mixed with 0.2 cc of each of the crystalline enzyme solutions† containing 5 mg per cm^3 in N/200 HCl (except ribonuclease which was made up in distilled water), the mixtures adjusted to a pH of 7.6 and placed in the refrigerator at 4°C for 24 hr, there was no apparent diminution in the virus content.‡ In fact, the infective titers of the digested virus appeared to be about 10 times higher than those of the undigested virus controls kept under the same conditions. Ultrafiltration of one sample of the digested material resulted in a reduction of about 99% of the original nitrogen content while the virus activity remained unaltered. On the basis of nitrogen content and virus concentration of the final product as compared to the original crude suspension, this procedure appeared to give the most satisfactory purification results thus far obtained.

Summary. While the lability of influenza virus limits the types of chemical and physical procedures to which it can be subjected, several methods are available for the purification of the virus. Either adsorption on calcium phosphate or ultrafiltration through collodion membranes results in preparations free of 90% or more of the nitrogenous material contained in the crude suspensions of infected mouse lungs. When these two techniques are combined, the residual virus recovered is freed of about 99% of the original nitrogen content. The loss of virus activity is only slight and its immunologic properties are apparently unimpaired.

Preliminary experiments with the proteolytic enzymes, trypsin, chymotrypsin and ribonuclease, indicate their possible use as a further step in the purification of influenza virus. A suspension of virus ultrafiltered after preliminary digestion contained but 1% of the original nitrogen, while the virus was concentrated tenfold.

† The crystalline enzymes made available by Dr. J. J. Bronfenbrenner were originally obtained from Dr. John Northrop.

‡ Control tubes consisting of 0.1% casein and enzymes, and mouse lung suspension and enzymes indicated that the enzymes were acting effectively under these conditions.

The author wishes to express his appreciation to Dr. J. J. Bronfenbrenner for his advice and suggestions and particularly for his generosity in making available the facilities of his laboratory at Woods Hole, Mass., where this work was begun during the summer of 1940.

13815

Age of Tissue in Agar-Slant Cultures and Multiplication of Virus of Herpes Simplex.

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The rate of multiplication and decline of a number of virus agents in association with living cells under various conditions of tissue culture has been studied by numerous investigators. The literature has been reviewed by Feller and his associates[1] who found that the quantity of vaccinia virus remained at or near its maximum in roller tube tissue cultures for at least 9 weeks, during which time the cells continued to live and to proliferate. Subsequently Enders and Florman[2] have shown that in suspended cell cultures of the Maitland-Rivers type this virus persists for about 4 weeks in association with viable cells. These observations are in contrast to those of Zinsser and Schoenbach[3] on the virus of Western equine encephalitis. In suspended cell cultures the titre of this agent reached its maximum within 3 days and then declined rapidly, more or less coincidentally with the decline in tissue metabolism. Pang and Zia[4] found that the maximal activity of the virus of St. Louis encephalitis on Zinsser's agar tissue medium was reached about the 6th day. In one case virus was detected as long as 36 days following inoculation although the titre was low from the 10th day on.

The experiments described in this paper were undertaken with the purpose of determining more precisely the relationship between the age of the tissue components in the agar-tissue medium and viral activity.

Materials and methods. The technique of cultivating the virus of herpes febrilis on Zinsser's agar slant tissue cultures has been previously described.[5] The same strain of virus was used in the present experiments. The brain of a moribund mouse was removed aseptically and ground in sand with the addition of 2 cc of broth. The resulting mixture was spun at 2800 rpm for 10 min and the clear supernatant fluid pipetted off. The intracerebral inoculation of an 0.04 cc amount into 24-day-old white mice resulted in death with typical signs within 36-72 hr. Inoculation of a series of agar slant tissue cultures was effected by permitting 0.2 cc of the supernatant fluid to flow over the tissue fragments spread on the surface of the agar slants. To transfer the virus to a fresh culture, 0.5 cc of Simm's solution[6] were added to an old slant and the tube gently rocked on its long axis for several minutes. During this process numerous small tissue fragments were washed down to the bottom of the tube. About 0.2 cc of this tissue fragment-containing fluid were then removed with a Pasteur pipette and added to an uninoculated tube. Titration of the viral activity of any given culture was carried out by scraping off the tissue fragments, placing them in a sterile mortar and then rinsing the tube with 2 cc of Simms' solution which were added to the contents of the mortar which likewise contained a small amount of sterile sand. After thorough grinding, followed by centrifugation at 2800 rpm for 10 min, the clear supernatant was pipetted off and tenfold dilutions made in infusion broth. Two mice were inoculated with each dilution. The highest dilution causing the death of both mice was taken as the end point.

1 Feller, A. E., Enders, J. F., and Weller, T. H., *J. Exp. Med.*, 1940, **72**, 367.

2 Enders, J. F., and Florman, A. L., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 153.

3 Zinsser, H., and Schoenbach, E., *J. Exp. Med.*, 1937, **66**, 207.

4 Pang, K. H., and Zia, S. H., *Chinese Med. J. Suppl.*, 1937, **3**, 446.

5 Cheever, F. S., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 113.

6 Sanders, M., *Arch. Path.*, 1939, **28**, 541.

TABLE I.

Age of culture in days at time of inoculation of virus	Additional days of incubation after inoculation	Age of culture in days at time of testing for viral activity	Ratio No. of mice dying to No. of mice inoculated
0	4	4	8/8
1	4	5	6/6
2	4	6	6/6
3	4	7	10/10
4	4	8	6/6
5	4	9	6/8
6	4	10	6/9
7	4	11	6/11
8	4	12	9/9
9	4	13	3/7
10	4	14	6/8
11	4	15	3/5
12	4	16	7/8
13	4	17	3/3
14	4	18	7/7
15	4	19	3/4
16	4	20	5/5
17	4	21	5/6
18	4	22	0/3
19	4	23	3/5
20	4	24	0/4
21	4	25	0/3

Autopsies were performed on mice dying with atypical signs, and cultures made of heart's blood and of emulsified brain. When possible, the latter material was inoculated into fresh mice. Deaths not obviously due to the herpes virus were classified as "no virus demonstrable."

I. *Survival of the virus on tissue cultures of different ages.* Agar slant tissue cultures were set up daily and incubated at 37°. After the first 24 hr the reaction in the tube became acid to phenol red due to the products of tissue metabolism. These tissue cultures of varying ages were then inoculated simultaneously with the same batch of virus, the tubes incubated for an additional 96 hr and then tested for the presence of the virus.

The results of several series of experiments are summarized in Table I.

Thus the virus survived for at least 4 days with a fair degree of regularity on tissue cultures as old as 17 days at the time the virus was inoculated.

Subsequently the activity of the virus on tissue cultures of varying ages was titrated. The results, summarized in Table II, indicated a fall from a titre of $10^{-3.5}$ in the case of virus inoculated into fresh tissue to 0 in that of virus inoculated into cultures of 18 days or older. The curve of viral activity, while showing a general downward course, failed to approximate a smooth line.

II. *Attempts to propagate the virus on tissue cultures of varying ages.* Since the virus could survive for 4 days at least on tissue as old as 17 days, we proceeded to determine whether cultures composed of aging cells would support the multiplication of the virus in serial transfer. Cultures were prepared at intervals of 4 days and stored at 37°. At the end of 12 days, fresh, 4-day-old, 5-day-old and 12-day-old cultures respectively were inoculated with the same batch of virus. The tubes were replaced in the incubator during an additional period of 96 hr and then removed. The material from each tube was transferred to a tissue culture 4 days younger, *i.e.*, the original fresh tissue culture now 4 days old was transferred to a fresh tissue culture; the original 4-day-old culture—now 8 days old—transferred to a 4-day-old tissue culture—etc. The viral activity of each tube was titrated at intervals of 8 days.

From the results graphically presented in Fig. 1, it is apparent that the virus can be propagated indefinitely on fresh tissue without significant loss of activity. In cultures

TABLE II.

Age of culture in days at time of inoculation of virus	Additional days of incubation after inoculation	Age of culture in days at time of testing for viral activity	Titre of virus
0	4	4	$10^{-3.5}$
1	4	5	10^{-3}
3	4	7	10^{-3}
7	4	11	10^{-2}
8	4	12	10^{-1}
10	4	14	$10^{-.5}$
12	4	16	10^{-2}
14	4	18	10^{-1}
15	4	19	10^{-1}
16	4	20	10^{-0}
17	4	21	10^{-1}
18	4	22	0
19	4	23	0
20	4	24	0
21	4	25	0

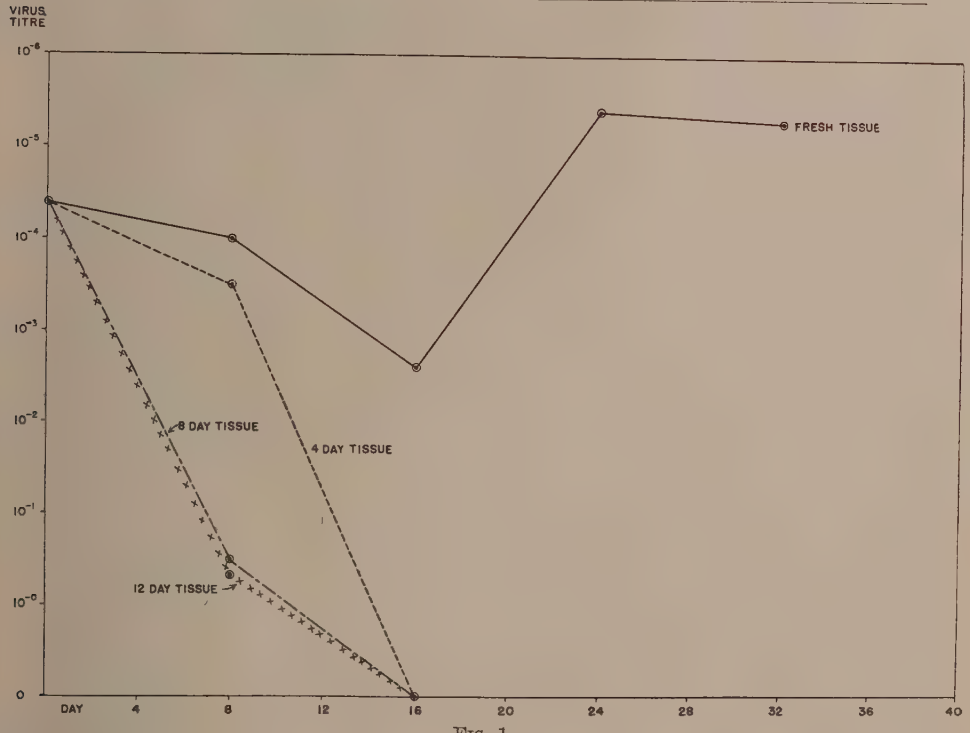

FIG. 1.

Behavior of virus of herpes simplex in serial passage on cultures composed of tissues of different age.

composed of older tissue, the titre of the virus declined in rough proportion to the relative age of the tissue culture. In no instance could the virus be demonstrated after the second passage, *i.e.*, when tested at 16 days.

Discussion. Since it has been shown[5] that the virus of herpes simplex does not survive for 96 hr at 37° if the tissue cells on the agar slants have been previously killed, it follows that some cells remained viable for as long as 17 days. In support of this conclusion, cultivation of fragments removed

from uninoculated agar cultures varying in age from 1 to 16 days have been found to result in the outgrowth of fibroblasts. A few observations on younger cultures inoculated 4 days previously with herpes virus also revealed fibroblastic proliferation when transferred to the plasma drop.

Accordingly we may be assured that the presence of living cells in agar slant tissue cultures as old as 17 days permits the survival of a portion of the virus for at least an additional 96 hr following inoculation. The titre, however, of viral activity of a culture after 4 days appears to vary approximately with its age at the time of inoculation. In this correlation between age of culture and titre of virus is to be found, we believe, the explanation for the fact that in freshly prepared cultures, when transferred at intervals of 4 days the virus may be propagated indefinitely without significant loss in titre, whereas the activity diminishes rapidly and soon disappears if cultures of 4 days or older are employed as the media for transfer.

These observations, then, represent further evidence in support of the intimate interdependence between the activity of the virus and the state of the tissue cell. In addition they suggest that multiplication of virus and survival may depend upon different cellular factors, since the former occurs only in the presence of cells freshly removed from the living embryo, but survival to a greater or less degree is afforded by cells whose vital functions have presumably been impaired in some manner. This conception has been stated by Plotz[7] and has received additional support in the experiments of Feller and his coworkers,[1] but the evidence presented here lends it greatly increased probability.

What the nature of such factors might be awaits further analysis. Multiplication of virus may depend upon the sum total of the normal metabolic processes of the cell as suggested by the experiments of Zinsser and Schoenbach.[3] Survival or preservation, on the other hand, may be correlated with the continued functioning of certain cellular enzymatic systems during the temporary suppression of others.

It is possible that the technique described of maintaining virus without multiplication through the use of pre-incubated tissue cultures may offer a means of investigating this problem, since it should permit observations of the effect on the virus and cells of the addition of various growth-promoting factors to the system.

Summary. 1. Agar slant tissue cultures as old as 17 days will permit the survival of the inoculated herpes simplex virus for at least 96 hr. 2. There appears to be a rough inverse relation between the age of the inoculated tissue culture and its virus content 96 hr later. 3. The virus may be propagated indefinitely on fresh agar slant tissue cultures; such is not the case if cultures 4 days or older are employed as the media for transfer.

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7 Plotz, H., *Compt. rend. Soc. Biol.*, 1937, **125**, 603, 719.

13816

Further Studies on Effect of Adrenal Cortex Extract and of Various Steroids on Capillary Permeability.*

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The writer has recently demonstrated that the extract of the adrenal cortex tends to inhibit the increased capillary permeability induced by either leukotaxine or an inflammatory exudate.[1] These observations have been interpreted as substantiating further the view that the hormone of the adrenal cortex perhaps plays a significant rôle in the regulation of fluid exchange as manifested by capillary filtration. These studies have been subsequently confirmed by Freed and Lindner.[2] The studies of the writer have primarily concerned themselves with the effect on capillary permeability of the adrenal cortex extract. At the time a footnote merely stated that preliminary observations had been undertaken with percorten, a synthetic preparation of desoxycorticosterone acetate.[1] This material seems to yield results somewhat similar to the effect elicited by the extract of the adrenal cortex. Freed and Lindner have failed to corroborate these preliminary observations on the inhibitory tendency of desoxycorticosterone acetate, although, as stated above, their own experiments with the crude extract of the gland have fully confirmed the writer's observations.[2] Fine and Fischmann have recently reported that desoxycorticosterone acetate does not seem to exert any beneficial effect in shock resulting from hemorrhage.[3] These investigators have been unable to obtain any inhibition of the seepage of trypan blue from the circulation into tissue when desoxycorticosterone acetate (percorten) is injected intravenously instead of introducing the steroid preparation locally. Their findings are not in the least surprising inasmuch as these workers have failed to follow the cutaneous route of injection of the hormone as originally described by the writer.[1] In general, the interpretation of Fine and Fischmann is open to doubt especially in view of their statement that trypan blue is not excreted by the kidneys.[3] The writer has frequently studied the urine of rabbits injected intravenously with trypan blue. The dye can readily be recovered in abundance in the urine.[4]

The various crystalline steroids of the adrenal cortex are soluble in oil. Freed and Lindner[2] merely suspended desoxycorticosterone acetate and corticosterone in physiological saline or in saline-gum acacia solution. It occurred to the writer that their negative results might very well have been referable to the insolubility in water of the preparations injected. However, since adrenal cortex extract is a solution, the results with this material, as obtained by these investigators, have fully confirmed our own original observations.[1] Furthermore, the writer has utilized a preparation of desoxycorticosterone acetate dissolved in sesame oil (percorten), whereas Freed and Lindner used the insoluble suspended material.† The present communication represents the results of observations which support the view that the adrenal cortex extract inhibits increased capillary permeability induced by leukotaxine or exudate. A similar effect is obtained with some of the steroid derivatives

* Aided by grants from the International Cancer Research Foundation, the Jane Coffin Childs Memorial Fund for Medical Research, and the Daland Fund of the American Philosophical Society.

1 Menkin, V., *Am. J. Physiol.*, 1940, **129**, 691.

2 Freed, S. C., and Lindner, E., *Am. J. Physiol.*, 1941, **134**, 258.

3 Fine, J., and Fischmann, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 98.

4 Menkin, V., unpublished studies.

† The synthetic preparation of desoxycorticosterone acetate, known as percorten, was obtained through the courtesy of Dr. Oppenheimer of the Ciba Pharmaceutical Products, Inc., in Summit, N.J.

TABLE I.
Inhibitory Effect of Various Compounds of the Adrenal Cortex on Induced Increased Capillary Permeability.

Rabbit No.	Accumulation of dye in control areas containing leukotaxine in sesame oil or exudate in sesame oil	Accumulation of dye into skin areas treated with leukotaxine or whole exudate in addition to: Desoxycorticosterone acetate in sesame oil (percorten)	Compound A (Kendall) in sesame oil	Compound B (Kendall) (corticosterone) in sesame oil	Compound E (Kendall) in sesame oil
21-89	++	trace	trace	0	trace to +
21-73	+++	0 throughout; faint trace at periphery	trace in center; + at periphery	trace to + primarily at periphery	++ to +++
21-88*	+		0		0
	++		0	+	0
21-94	+++	+			
3-56	++ to +++	0 throughout; + at periphery			
20-65†	trace to +	0			
20-22‡	++	0			
	+++	0 in center; ++ at periphery			
20-56§	++	0			
	++++	++			

*In this animal the accumulation of dye in the cutaneous areas was negligible but fairly adequate readings were made in the subcutaneous region.

†Final readings made within 17 minutes, whereas in all other experiments the time of observation lasted from a half hour to one hour when the final readings were recorded.

‡Leukotaxine dissolved in saline in this experiment instead of emulsifying with sesame oil as in the other experiments.

§Whole exudate instead of leukotaxine utilized in this experiment.

of the adrenal cortex provided that these compounds are first dissolved in a suitable medium, such as sesame oil. If, however, they are suspended either in saline or serum there follows no inhibitory tendency to increased capillary permeability.

The technique of cutaneous injection and of intravenous trypan blue administration has been described previously.[1] Leukotaxine was isolated from inflammatory exudate.[5] 11-dehydrocorticosterone (Compound A), corticosterone (Compound B), and 17-hydroxy-11-dehydrocorticosterone (Compound E) were obtained through the courtesy of Dr. E. C. Kendall.[6] Percorten, a soluble preparation of desoxycorticosterone acetate in sesame oil, was obtained from Ciba. In accordance with the kind suggestion of Dr. E. C. Kendall, Compounds A, B, and E were brought into solution by adopting the following procedure: Approximately 1 to 2 mg of each fraction was dissolved in a half cm^3 of absolute alcohol. This was followed by the addition of a half cm^3 of sesame oil. The vials with the material were then placed in a vacuum oven and the contained alcohol evaporated at room temperature. About 2.5 mg of leukotaxine or 0.3 cc of exudate was then carefully suspended in the sesame oil containing the steroid fraction. The mixture was injected intracutaneously in the skin of the abdomen of a rabbit, and the experiment carried out as previously described.[1] Cutaneous areas were also injected with a similar amount either of leukotaxine suspended in sesame oil or of exudate emulsified with this medium. These areas served as controls to the ones containing in addition the fractions of the adrenal cortex. 2.5 mg of percorten or desoxycorticosterone acetate dissolved in sesame oil was also employed in a number of experiments.

The results are summarized in Table I. It is quite clear that desoxycorticosterone acetate, Compounds A, B, and E dissolved

[5] Menkin, V., *Dynamics of Inflammation*, Macmillan Company, New York, 1940.

[6] Kendall, E. C., *J. Biol. Chem.*, 1940, **133**, Li (Proc. Am. Soc. of Biol. Chem., Mar., 1940); *Arch. Path.*, 1941, **32**, 474.

TABLE II.
Effect of Adrenal Cortex Extract and of Some Aqueous Insoluble Crystalline Derivatives on Induced Increased Capillary Permeability

Rabbit No.	Accumulation of dye in control areas containing either (1) leukotaxine in saline or serum; or (2) exudate + saline	Accumulation of dye into skin areas treated with leukotaxine or whole exudate in addition to: Extract of adrenal cortex	Compound B (Kendall's corticosterone) suspended in saline	Compound E (Kendall) suspended in saline	Desoxycorticosterone acetate suspended in saline or serum
21-63	+++	0	++ to +++	++ to +++	
21-77	+	0	++	++ to +++	
21-78	+		+		
21-79	+++				+++
20-43*	++				++; ++; ++ (3 areas tested)
24-69	+	0			
21-38†	++++	faint trace			
21-95‡	++ to +++	0			
21-97‡	+++	0			

*Materials suspended in serum instead of physiological saline.
†Extract of adrenal cortex obtained from Wilson Company; in all other experiments the extract was furnished by Dr. E. C. Kendall.
‡Whole exudate employed instead of leukotaxine.

in sesame oil tend to inhibit the marked increase in capillary permeability induced by leukotaxine or exudative material.

On the contrary, desoxycorticosterone acetate crystals‡ suspended in serum or saline; or compounds B and E likewise suspended in saline in concentrations approximating those described above failed to inhibit the enhanced capillary permeability induced by leukotaxine. The results are conveniently summarized in Table II. Furthermore, it is also clear that in contrast to the insoluble steroid preparations, the crude extract of the adrenal cortex, as found previously, markedly inhibits capillary permeability.[1] In general, the effect on the reduced filtration of dye through the capillary wall is definitely more pronounced with the adrenal cortex extract than with the use of crystalline steroids dissolved in oil. (*cf.* Tables I and II).

Conclusions. The extract of the adrenal cortex inhibits the increased capillary permeability induced either by an inflammatory exudate or by leukotaxine, in turn isolated from exudative material. A similar tendency, although generally not quite as pronounced, can be elicited by various steroid fractions such as synthetic desoxycorticosterone acetate (percorten) or Kendall's Compounds A, B, and E derived from the adrenal cortex, provided that these substances prior to assay are dissolved in an oily vehicle. Mere suspension of the steroids in saline or serum fails to reveal any inhibitory activity on capillary permeability.

‡ Obtained through the courtesy of the Research Division of the Schering Corporation, Bloomfield, N.J.

13817 P

Evaluation of Nicotinic Acid Nutrition by Studies of Urinary Excretion.

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The adequacy of nicotinic acid nutrition in man is determined at the present time solely on the basis of clinical findings. Several investigators[1,2,3] have reported that there is little difference in the quantity of nicotinic acid and its derivatives excreted in the urine in a 24 hr period by normal persons and by patients with dietary deficiency. Perlzweig, Sarett and Margolis[2] have recently suggested that the urinary excretion following the administration of 500 mg of nicotinamide may serve as a test of nicotinic acid deficiency.

This report deals with observations made on 6 normal persons, 18 hospital patients without signs of deficiency disease and 10 patients with clinical findings indicating deficiency of one or more members of the B group of vitamins. Of this last group 7 had lesions of pellagra, 5, evidence of ariboflavinosis and one, thiamin deficiency. The quantity of nicotinic acid and its derivatives excreted in the urine in a 24 hr period was determined by the method of Perlzweig, Levy and Sarett[4] with minor modifications.[5] Nicotinic acid is excreted in a number of forms, a small percentage as nicotinamide, coenzyme and nicotinuric acid (acid hydrolyzable derivatives) and a large percentage as trigonelline, which must be determined separately. The method is very satisfactory for the determination of acid hydrolyzable derivatives and is the best available for the estimation of trigonelline, although in our experience only 40 to 50% of this compound added to urine can be recovered.

Since trigonelline is the main excretion product of nicotinic acid and since it is present in many foods, it is essential in studying nicotinic acid metabolism to place subjects on a diet low in trigonelline, which was done in this investigation.

In the 34 subjects studied, there was no difference in the amount of "nicotinic acid"† excreted in the urine in 24 hr by normal persons and by patients with and without clinical evidence of deficiency of the B vitamins. (Table I). The average output of trigonelline, however, was highest in the normal group, next in the patients without evidence of deficiency disease and lowest in patients with pellagra, ariboflavinosis and thiamin deficiency. The total 24 hr output of nicotinic acid derivatives averaged in normal persons 16.2 mg, in patients with deficiency 8.7 mg. While these findings suggest that there is a decreased excretion of trigonelline in nicotinic acid deficiency there is marked overlapping in the range of values in the 3 groups. The status of nutrition in a given patient therefore, cannot be determined by measuring the excretion in the urine for 24 hr.

Sixteen persons were given 300 mg of nicotinamide‡ orally and the quantity of nicotinic acid derivatives excreted in the urine during the subsequent 6 and 24 hr determined. Nicotinamide is excreted almost entirely as trigonelline (Table II). Normal persons excreted on an average twice as much trigonelline as did hospital patients in both

* Aided in part by a grant from Eli Lilly & Co. to the Department of Tropical Medicine, Tulane University School of Medicine.

1 Sarett, H. P., Huff, J. W., and Perlzweig, W. A., *J. Nutrition*, 1942, **23**, 23.

2 Perlzweig, W. A., Sarett, H. P., and Margolis, L. H., *J. Am. Med. Assn.*, 1942, **118**, 28.

3 Field, H., Melnick, D., Robinson, W. D., and Wilkinson, C. F., *J. Clin. Invest.*, 1941, **20**, 379.

4 Perlzweig, W. A., Levy, E. D., and Sarett, H. P., *J. Biol. Chem.*, 1940, **136**, 729.

5 Dann, W. J., and Handler, P., *J. Biol. Chem.*, 1941, **140**, 201.

† The term nicotinic acid is used to include all acid hydrolyzable derivatives.

‡ Nicotinamide furnished through the courtesy of Merck & Co.

TABLE I.
Urinary Excretion of Nicotinic Acid Derivatives in 24 Hours in Subjects on a Diet Low in Trigonelline.

Subjects		Nicotinic acid* (mg)	Trigonelline† (mg)	Total (mg)
Normal persons (6)	Range	0.9-1.5	11.1-21.1	12.3-22.1
	Avg	1.2	15.0	16.2
Patients without deficiency (18)	Range	0.6-3.0	2.5-25.2	3.5-26.6
	Avg	1.4	8.6	10.0
Patients with "B" deficiency (10)	Range	0.5-1.6	3.8-14.0	4.3-15.0
	Avg	1.0	7.7	8.7

*Acid hydrolyzable derivatives.
†Expressed as amount determined as nicotinic acid.

the 6 and 24 hr periods after the test dose. There was little overlapping of findings in the 2 groups. The output of trigonelline was slightly less in patients with vitamin B complex deficiency than in the other patients. The diet in all the patients had been somewhat inadequate. Since approximately 50% of the trigonelline excretion occurred in the first 6 hr after the test dose, as much information was obtained by measuring the 6 as the 24 hr output.

The extra excretion after the administration of 300 mg of nicotinamide was calculated by subtracting the total output in a control period of 24 hr from that found on the day of the test dose. Normal persons excreted 18 to 76 mg, hospital patients 0 to 41 mg, and patients with vitamin deficiency, 6 to 21 mg. The retention of nicotinamide appeared to be closely related to the degree of dietary deficiency.

Determination of the amount of trigonelline excreted in a 6 hr period after the administration of 300 mg of nicotinamide may serve as an index of nicotinic acid nutrition.

TABLE II.
Urinary Excretion of Nicotinic Acid Derivatives After the Oral Administration of 300 mg of Nicotinamide.

Test performed		Normal persons (6) Range	Avg	Patients without deficiency (6) Range	Avg	Patients with "B" deficiency (4) Range	Avg
Excr. after test dose 6 hr (mg)	N.A.*	0.8- 4.3	2.5	0.6- 4.8	2.0	0.2- 1.0‡	0.6‡
	Trig.†	17.1-39.6	25.9	7.5-17.8	13.0	10.1-14.7‡	12.4‡
	Total	18.7-43.9	28.4	8.1-22.6	15.0	10.3-15.7‡	13.0‡
Excr. after test dose 24 hr (mg)	N.A.*	2.0- 4.5	3.3	1.0- 7.4	3.4	0.8- 2.0	1.2
	Trig.†	27.3-83.9	49.6	17.4-39.7	27.1	11.1-31.5	20.4
	Total	30.1-88.4	52.9	19.2-47.1	30.5	11.9-33.5	21.6
Extra excr. after test dose (mg)	Total	17.8-76.1	36.7	0.0-40.7	21.7	5.6-20.5	13.7

*N.A—Nicotinic acid.
†Trig.—Trigonelline.
‡Six-hour output measured in only 2 patients.

13818

Toxicity of Acetates for Rats.

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In investigations of the intermediary metabolism of acetate using the heavy carbon isotope as a tracer element it became necessary to know what acetate doses could be tolerated by rats for a two-week period. While a number of investigators, including Deuel and Milhorat,[1] Taistra,[2] Daken,[3] Lusk,[4] McKay, Barnes, Carne and Wick,[5] Lamb and Evvard,[6] have given what may be considered massive doses of acetates without lethal effects, there are few reports of lethal effects produced by acetates. Recently Woodward, Lange, Nelson and Calvery[7] determined the acute toxicity of sodium acetate for rats and found a minimum lethal dose of 55 millimols per kg. Sollman[8] added acetic acid to the drinking water of rats and found that with an intake of less than 3.5 millimols per kg per day from drinking water of 0.25% acetic acid there were no significant disturbances of weight or appetite. Higher dosages reduced both. There is no information in the literature on toxic doses administered over time periods of 7 to 14 days. Nor is there information on relative toxicities of acid, sodium and ammonium salts. For metabolic investigation this information is necessary.

Experimental. Female rats usually in the weight range of 170-200 g were used. They were raised on a stock diet of casein 15, whole milk powder 10, NaCl 0.8, $CaCO_3$ 1.5, butter 5.2, whole ground wheat 67.5, cod liver oil 1.0. The rats were allowed free access to this diet and water throughout the experiments. During the experimental period they were given by stomach tube a 1.0 N solution of the acetates twice a day with doses 8 hr apart. Daily weighings were made and the physical condition of the animals noted.

Results. The summarized results are given in Table I. Data for each rat are given in column 2 where the daily dosage in millimols per kg is given in parentheses followed by the number of days survival for each rat. In the right hand column of the table there is calculated the percentage of the total metabolic rate contributed by acetate combustion assuming a normal metabolic rate of 30 calories per day per 200 g rat.

The results of the table indicate that a dosage of 30 millimols per kg of acetic acid can be tolerated for a 2-week period while 40 millimols per kg day will cause death in 3 to 5 days usually. Sodium acetate is considerably less toxic, the maximum tolerable dose being 70 to 80 millimols per kg per day. Ammonium acetate of 60 millimols per kg day is the maximum dosage which can be tolerated by rats for a 2-week period.

Discussion. It is to be noted that when high doses were used the mortality was high on the second, third or fourth day. If the animal could survive the period there was a good possibility of surviving the 14-day period. For investigations on metabolism of acetates using heavy carbon the procedure is recommended of starting with a high dosage of ordinary acetate for 7 days before using the rare material on the survivors.

Deuel and Milhorat[1] and MacKay,

1 Deuel, H. J., and Milhorat, A. T., *J. Biol. Chem.*, 1928, **78**, 299.

2 Taistra, Sophie A., *J. Biol. Chem.*, 1921, **49**, 479.

3 Dakin, H. D., *J. Biol. Chem.*, 1907, **3**, 57.

4 Lusk, Graham, *J. Biol. Chem.*, 1921, **49**, 453.

5 MacKay, E. M., Barnes, R. H., Carne, H. O., and Wick, A. N., *J. Biol. Chem.*, 1940, **135**, 157.

6 Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, 1919, **37**, 329.

7 Woodward, G., Lange, S. W., Nelson, K. W., and Calvery, H. O., *J. Ind. Hyg. Tox.*, 1941, **23**, 78.

8 Sollman, Torald, *J. Pharm. Exp. Therap.*, 1920-21, **16**, 463.

TABLE I.
Survival Times and Weight Changes of Rats Receiving High Dosages of Acetate.

Substance fed	(Daily dosage millimols per kg), No. of days survival	% metabolic rate due to acetate combustion
Acetic Acid	(30) 14, 14, 14; (40) 2, 2, 3, 3, 3, 3, 4, 4, 5, 5, 14, 14; (50) 2, 2, 3, 3, 3, 3, 4, 5, 5, 5, 6, 6; (60) 2, 2, 2, 2	4.2 to 8.4
Sodium Acetate	(30) 4, 14, 14; (40) 8, 13, 14; (50) 14, 14, 14; (60) 14, 14, 14; (70) 14, 14, 14; (80) 2, 14, 14; (90) 1, 3, 7, 14, 14; (100) 1, 2, 2, 14; (110) 1	4.2 to 15.4
Ammonium Acetate	(30) 14, 14, 14; (40) 8, 14, 14; (50) 4, 14, 14; (60) 1, 14, 14; (70) 1, 1, 4	4.2 to 9.7
$NaHCO_3$	(100) 14, 14, 14; (150) 1, 1, 1	
Water	14 (volume equal to largest dosage)	

Barnes, Carne and Wick[5] have reported gastrointestinal irritation with acetic acid and have used preferably sodium acetate. A difficulty in the use of the sodium salt is that excess sodium remains after combustion of the acetate radical and alkalosis develops. Alkalosis, however, is not the toxic factor since a dosage of 100 millimols per kg-day of NaOAc produced death in 1 to 2 days of 3 of a group of 4 rats while 100 millimols per kg-day of $NaHCO_3$ produced no fatalities in a group of 3 rats in 14 days. Alkalosis due to the sodium salt can be prevented by using the free acid or the ammonium salt. These two acetates while not producing alkalosis are both irritant to the alimentary tract and probably produce their toxic symptoms by injury to this organ.

When sodium acetate is given in 2 daily doses 70-80 millimols per day can be tolerated for 14 days. The total daily tolerable dose exceeds the single toxic dose of 55 millimols as found by Woodward, Lange, Nelson and Calvery.[7]

The main toxic symptoms observed just before death were loss of weight, blistering of the paws, and reddish appearance of nose and whiskers.

13819

Isolation of *Staphylococcus albus* from Hemolymph of the Roach, *Blatta orientalis.*

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Previous examinations of hemolymph from the oriental roach, *Blatta orientalis,* have demonstrated that bacterial infections of the body fluid are associated with high total hemocyte counts[1] and high percentages of mitotically dividing hemolymph cells.[2] Since it is known that some insect leucocytes are phagocytic, the above responses may be interpreted as a mechanism whereby the numbers of hemocytes are increased for defense purposes. Thus a larger number of ingesting phagocytes would be available for combatting the invading organism. In addition, other symptoms indicated that presence of

1 Tauber, Oscar E., and Yeager, J. F., *Ann. Ent. Soc. Am.,* 1935, **28**, 229.

2 Tauber, Oscar E., *Ann. Ent. Soc. Am.,* 1940, **33**, 113.

the bacteria was decidedly harmful. Although occurrence of bacteria in insects has been noted many times,[3] descriptions often have been inadequate and identification impossible. Therefore, in this case, samples of infected hemolymph were carefully taken from a cut antenna, and transferred to various bacteriological media. Later, single organisms were isolated and cultivated in pure culture. The following characteristics were observed after 24 to 48 hr incubation:

Morphology: Spheres, 0.55 to 0.75 μ occurring singly or in irregular groups. Gram positive.

Agar colonies: Raised, circular, smooth, porcelain-white, glistening, entire.

Agar slant: Moderate growth, filiform, smooth, glistening.

Blood agar: β-hemolysis.

Broth: Turbid, with rather coarse, granular sediment.

Gelatin stab: Slow, saccate liquefaction.

Litmus milk: Slightly acid, with tendency to coagulate.

Potato: Thick, smooth, white glistening growth. Brown with age.

Indol: Not produced.

Nitrates: Reduced to nitrites.

Hydrogen sulphide: Produced.

Carbohydrates and alcohol fermented: Acid in dextrose, lactose, sucrose, and mannitol; negative in inulin and raffinose.

Oxygen relations: Aerobic and facultative.

Optimum temperature: 30°C.

When the above characteristics were checked in Bergey's Manual,[4] the isolated causative agent was found to compare closely to *Staphylococcus albus*.* The lower optimum temperature (Bergey gives 37°C) may indicate the development of a strain of *albus* which has become accustomed to growth in a poikilothermic animal, rather than in or on a homeothermic form.

Pathogenicity of the described organism for the oriental roach was shown by various symptoms. Food was taken in small quantities, if at all, especially when the infection was severe. Slow and feeble movements pointed to a general body weakness. If placed on its dorsal surface, an afflicted specimen was often unable to right itself. Righting movements were uncoördinated and ineffective. Respiratory movements were irregular. Evidence of a neurotoxin manifested itself in a progressive anteriorly-moving paralysis. Metathoracic legs were affected first. If the animal was stimulated to move, these legs were useless and were dragged along. As the mesa- and prothoracic pairs became useless, spasmodic twitchings of the whole body also appeared. In the final stages of the disease, the legs were folded under the body; the head was tucked beneath the forelegs; and the whole animal became arched. This posture was maintained until death.

In addition to these external signs, and the increase in hemocyte mitosis, the circulating cells became filled with vacuoles. Many hemocytes underwent cytolysis. During the final stages of the affliction, affected hemolymph became thick and milky from the presence of tremendous numbers of parasitic bacteria.

Just how the organism made its initial entry into the roaches is not known. Infections were already established when the insects were trapped. However, transfer of the causative agent was easily accomplished by needle inoculation of normal roaches with diseased hemolymph, or with a broth culture of the isolated causative agent. After the disease became established, recovery of the organism was always possible. In all cases of successful inoculation, the roaches died from the effects of the affliction.

The incidence of this infection among roaches of this species is not known. All original infected specimens came in a shipment from a collector in Mississippi. Two other groups, totaling more than 500 individuals, from other localities, gave no evidence of the presence of the organism.

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[3] Paillot, A., *L'infection chez les insectes. Immunité et symbiose*, Patissier, Paris, 1933.

[4] Bergey, D. H., Breed, R. D., Murray, E. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, Williams & Wilkins, Baltimore, 1939.

* Identification of the organism was made by Dr. J. R. Porter, Department of Bacteriology, College of Medicine, State University of Iowa, Iowa City.

Summary. A bacterium, pathogenic for the roach, *Blatta orientalis,* was isolated in pure culture from the hemolymph of this insect, and proved to be *Staphylococcus albus,* as described in Bergey's Manual. Infections could be established in normal roaches by inoculation with diseased hemolymph or with broth cultures. Death was preceded by a characteristic progressive paralysis.

13820

Protective Action of Sulfanilamide Against Liver Cirrhosis from Chronic Poisoning with Carbon Tetrachloride.*

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In a previous article[1] it was reported that oral administration of sulfanilamide, sulfathiazole or sulfapyridine prior to exposure reduced markedly the mortality of rats following acute poisoning by inhalation of carbon tetrachloride. Additional work on this problem dealing with the effect of sulfanilamide against chronic poisoning from carbon tetrachloride, as well as results obtained by use of other possible protective substances, is presented at this time.

These chronic poisoning experiments confirmed our previous observations. In preliminary experiments in which sulfanilamide was incorporated in the diet, (100 mg per 100 g of food) it was found that the animals ate well when no carbon tetrachloride was administered, but as soon as the periods of anesthesia were begun they practically refused to eat and consequently became more susceptible than the controls. This necessitated the administration of the drug by stomach tube in all subsequent experiments. One cm^3 of solution per 100 g of body weight was always used, the drug being either completely dissolved or with undissolved material in fine suspension in the water. Approximately 3 hr were allowed to elapse from the time of sulfanilamide administration before each period of anesthesia was begun. A summary of the experimental results is presented in Table I. It will be seen that there is a certain amount of overlapping but the general protective tendency is evident. In experiments 4 and 5, records of the daily food consumption were made. During the first few weeks no significant difference was noted between control and treated animals, but as the experiment progressed the food consumption of those receiving sulfanilamide was definitely greater than that of control animals.

The possible protective action of a variety of other substances has been studied in a number of acute poisoning experiments, but no protective action was obtained in any case. These substances included the following vitamin preparations, thiamine hydrochloride, riboflavin, pyridoxin hydrochloride, calcium pantothenate, nicotinic acid, inositol, para-aminobenzoic acid and ascorbic acid, and the following amino acids, *dl*-alanine, glycine, *l*-tyrosine and *dl*-methionine. Since para-aminobenzoic acid is known to inhibit the action of sulfanilamide against microorganisms, acute poisoning experiments were carried out in which this vitamin was administered simultaneously with sulfanilamide. No evidence of inhibition of the protective action of the sulfanilamide was obtained.

Discussion. The mechanism for the protective action of the sulfonamide drugs against carbon tetrachloride is obscure. Mackenzie and Mackenzie[2] have recently reported that

* Acknowledgment is gratefully made to the John and Mary R. Markle Foundation for its financial aid.

1 Leach, B. E., and Forbes, J. C., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 361.

2 Mackenzie, J. B., and Mackenzie, C. G., *Proc. Fed. Am. Soc. f. Exp. Biol.*, 1942, **1**, 122.

TABLE I.
Effect of Sulfanilamide on Production of Liver Cirrhosis from Chronic Carbon Tetrachloride Poisoning.

Exp. No.	No. of rats showing liver cirrhosis†				No. died	Remarks
	None	Mild	Moderate	Severe		
1	8	3	1	0	0	7.5 mg* sulfanilamide daily. CCl_4 daily except Sundays for 22 days, then every other day for 25 days.
	0	0	1	3	8	Controls for above.
2	6	0	0	0	2	7.5 mg* of drug daily for 54 days. CCl_4 daily for 14 days, then 3 or 4 times a wk for 40 days.
	0	3	5	1	5	Controls for above.
3	4	4	1	0	0	7.5 mg* of drug daily for 52 days. CCl_4 daily for 8 days, then every other day for 44 days.
	0	3	3	0	2	Controls for above.
4	2	4	1	1	0	30 mg* of drug every other day for 15 days, then 3 times per wk for 32 days. CCl_4 given 3 hr after each sulfanilamide adm.
	0	0	3	0	7	Controls for above.
5	5	4	0	0	0	30 mg* of drug 3 times per wk for 75 days. CCl_4 given 3 hr after each sulfanilamide adm.
	2	2	3	2	4	Controls for above.

*Per 100 g of body weight, administered by stomach tube.
†The grading of cirrhosis was determined as follows:
Mild = Definite round cell infiltration with increased connective tissue but no pseudo-lobule regeneration.
Moderate = Definite and numerous pseudo-lobule regeneration with much intervening connective tissue.
Severe = Entire liver made up of pseudo-lobules with marked and interlobular scarring.

certain sulfonamide drugs cause marked enlargement of the thyroid gland in rats, with loss of colloid material and reduction in the basal metabolic rate. Administration of crystalline thyroxine to rats has been shown by McIver[3] to increase their susceptibility to chloroform. The possibility exists that the protective action of sulfanilamide against carbon tetrachloride might be due to inhibition of thyroid activity. Experiments to test this possibility are contemplated for the near future.

Summary. Administration of sulfanilamide to rats retards the development of liver cirrhosis from chronic poisoning with carbon tetrachloride. A number of vitamins and amino acids were tested for possible protective action in acute poisoning experiments, but none was found to increase the animal's tolerance. Administration of para-aminobenzoic acid did not inhibit the protective action of sulfanilamide against acute poisoning by carbon tetrachloride.

[3] McIver, M. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 201.

13821 P

Comparative Toxicity of Three Forms of Vitamin D for Albino Rats.

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There are two views as to the nature of the toxic action of Vitamin D.[1] One is that toxication is a function of the antirachitic unitage, while the other holds that this result depends either on toxic by-products of activation or on end products of metabolism of the vitamin in the body.

In the course of some studies on the alleged hypertensive action of Vitamin D in rats[2] it appeared that different preparations of the vitamin gave different degrees of toxication. Consequently, 3 groups of rats were selected from our standardized stock colony at ages ranging from 242 to 601 days. One of 3 preparations of Vitamin D was administered to each group in daily doses of 75 units per g of body weight.

Group 1, comprising 4 males and 5 females received Vitamin D_3 (activated 7 dehydrocholesterol in sesame oil),* by stomach tube.

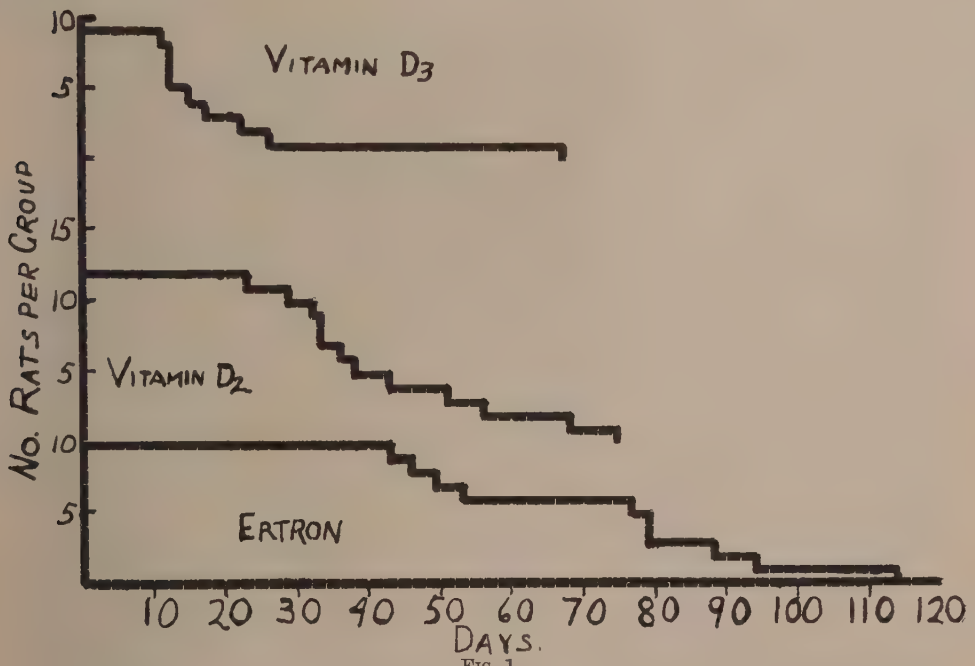

FIG. 1.

Survival rates of comparable groups of rats receiving identical antirachitic unitage of three different preparations of Vitamin D.

Group 2, six males and 6 females received Vitamin D_2† (calciferol in corn oil) by stomach tube.

Group 3, seven males and 4 femlaes, received Vitamin D in the form of ertron, an electrically activated ergosterol.‡ This ma-

1 Reed, C. I., Struck, H. C., and Steck, I. E., *Vitamin D*, 1939, University of Chicago Press.

2 Mrazek, R., Novak, C. G., and Reed, C. I., *Proc. Am. Physiol. Soc.*, April, 1942.

* Courtesy of Dr. J. Waddell, DuPont Co.

† Courtesy, Winthrop Chemical Co.

‡ Courtesy, Nutrition Research Laboratories.

terial was not purified, but the activated stock mix was dissolved in corn oil and given by stomach tube.

It is emphasized again that the dose of each preparation was 75 international antirachitic units per g of body weight. There was no standardization of toxic factors, if such exist.

The duration of life in each group is shown graphically in Fig. 1, each unit space representing one rat and each fall, therefore, represents the death of one animal.

In Group 1, the first rat was found dead on the 11th day, and the 8th on the 26th day; the 9th rat survived to the 67th day. This last survivor was a male, but ordinarily no distinction could be made between the two sexes in survival time. The average period of survival was 29.5 days and 15.4 days respectively.

In Group 2, the first rat, (m), died on the 23rd day, while the last (f) died on the 75th day. The average survival period for males was 43.3 days, for the females, 42.3 days.

In Group 3, the first rat (f) died on the 43rd day, the 10th (f) on the 94th day, and the 11th (m) on the 114th day. The mean survival time was 68.4 days for males, 71 for females.

Despite the small numbers of animals in each group it appears that the differences in toxic responses to the different preparations may be specific. Since the antirachitic dose of each preparation was the same, it must be concluded that the fatal outcome of toxication was conditioned, at least, by some other factor not expressed by antirachitic units. Post mortem examination did not reveal any differences of significance in the type of lesions.

13822 P

Effects of Various Chemical Agents on Survival of Primative Respiratory Mechanism.

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That young animals are much less susceptible to anoxia than are adults has been well established.[1-5] Selle and Witten[4] have shown that the respiratory center of rats, rabbits, cats and dogs is itself much more tenacious and viable in the young than in the adult, and that until approximately 6 weeks of age the survival of the respiratory mechanism of the rat is inversely proportional to age. Following decapitation or ligation of the cerebral vessels, the isolated ischemic head of a full grown rat gasps 5 to 8 times over a period of 10 or 20 sec and thereafter remains motionless; in a week old animal the phenomenon continues for 30 min or more.

Unlike adults, rats under 6 weeks of age display 2 periods of respiratory movements: an initial series which consists of 6 to 12 gasps and lasts 20 to 30 sec, and a second series which begins 30 to 50 sec after cessation of the initial or first series and lasts for a variable time depending upon the age. The total number of gasps in both series, as well as the total duration of gasping, is greater the younger the animal. The second series, which is influenced most by age, lasts 30 to 40 min in new-born animals. As age increases, the number of gasps, as well as duration of gasping, diminishes uniformly. Similar observations have been made on

1 Reiss, M., and Haurowitz, F., *Klin. Wchnschr.*, 1929, **8**, 743.

2 Avery, R. C., and Johlin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1184.

3 Kabat, H., and Dennis, C., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 534.

4 Selle, W. A., and Witten, G. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 495.

5 Himwich, H. E., Alexander, F. A. D., and Fazekas, J. F., *Am. J. Physiology*, 1941, **134**, 281.

ischemic heads of rabbits, cats and dogs.

The present study was designed to determine the effectiveness of certain drugs on the activity of this primitive respiratory mechanism. Using a technique previously described[4] for determination of survival of this mechanism, a large number of chemical agents (67) varying widely in composition and action, were injected subcutaneously or intraperitoneally into 12 - 15-day-old rats (weight 18-26 g). Following an interval ranging from 5 min to several hours, or even days, depending upon the rapidity of action of the test material, the head was quickly isolated. The gasps resulting were mechanically recorded. In a few experiments the animals were given anesthetic gases, rather than hypodermic injections, prior to decapitation.

Control animals consisted of an equal number of 12 - 15-day-old litter mates weighing 18-25 g. Such animals show a uniform respiratory pattern of 23 to 30 gasps following onset of anoxia, there being a relatively constant number of gasps in the first series (8) and a variable number in the second series (15-22). The survival time is 3-6 min and is influenced, as is the number of gasps of the second series, by litter characteristics and by the nutritive condition and degree of hydration of the animals. Untreated litter mates subjected to identical conditions yield remarkably uniform results. In the present series the variation in survival time of the controls was about 8%, the variation in the number of gasps was 4.8%. The results for the test animals were compared with those of the litter mate controls which were given a similar volume of the vehicle (usually .9% NaCl) used in the experimental animals. Deviations from control values were expressed in per cent.

Most of the chemical agents tested had little or no effect on the nature or character of the gasping pattern or on the total duration of gasping unless given in toxic amounts, in which case the survival period was greatly decreased. In a number of instances, however, definite changes in the character and number of gasps, as well as the total duration of gasping, were observed with non-toxic doses. Table I gives the results for some of the more effective drugs tried, together with the dosage used and the time thereafter at which the effect of the drug was noted.

Outstanding among the agents which reduced the survival time and the number of gasps are iodoacetic acid, thyroxin, dinitrophenol, ether, chloroform and hypnotics of the barbituric acid series. Agents which have been found to increase the total number of gasps and the duration of gasping are morphine, alcohol, chloralose, urethane and cyclopropane. With exception of morphine, the latter increased the survival time by 150 to 280%, and the number of gasps of the second series by 100 to 280%. Morphine brought about increases of about 50%. The character of gasps in the second series was markedly altered by these drugs. In general,

TABLE I.
Influence of Various Chemical Agents on Survival of the Isolated Heads of 12-15-day-old Rats.

Chemical	Dose	Interval after drug given	No. of Animals	Respiratory activity based on avg control values: Total No. gasps %	Survival time %
1. Iodoacetic Acid	.05 cc 1%	8 min.	10	decreased 82	decreased 85
2. Ether	5% in air	5 "	11	" 77	" 80
3. Chloroform	2% " "	5 "	8	" 75	" 85
4. Nembutal	.1 cc 1%	8 "	16	" 64	" 65
5. Dinitrophenol	.3 cc 1%	30 "	17	" 58	" 68
6. Thyroxin	.05 mg daily	8 days	10	" 20	" 52
7. Morphine	2 mg	1 hr	12	increased 54	increased 49
8. Cyclopropane	25% in air	5 min	13	" 170	" 78
9. Alcohol	.5 cc 20%	15 "	15	" 265	" 156
10. Chloralose	.1 cc 1%	15 "	12	" 270	" 280
11. Urethane	.4 cc 4%	15 "	13	" 280	" 250

the strength or amplitude of the mandibular movements was somewhat reduced in the early phase of the second series. The duration of individual gasps was always shorter the greater the frequency of gasping.

Although the total expenditure of energy has not been accurately determined, it is apparent that morphine, alcohol, urethane, chloralose and cyclopropane definitely increase the over-all energy liberation. The mechanism involved in this additional energy liberation is being studied.

13823

Ejaculation Induced by a Uterine Drug (Gravitol)

GEORG BARKAN.* (Introduced by Sanford B. Hooker.)

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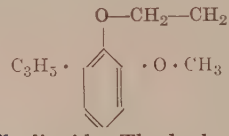
Gravitol is the di-ethyl-amino-ethyl-ether of 2-methoxy-6-allyl-phenol. The base itself



is a thin oily liquid. The hydrochloride is a crystalline water-soluble substance. For the purposes of this investigation the 1% aqueous solution of the hydrochloride was used. Gravitol, also on the market with the trade names Clavitol and Uterol, has been introduced as a uterine drug.[1] Significant stimulating as well as depressing actions upon uterus, intestine, blood vessels and blood pressure were previously described by Barkan and his associates.[2–6] Pharmacological observations were also reported by Anan[7] and Okazaki.[8] A discussion on the drug's action upon the heart went on between van Dongen and Bijlsma[9–11] on the one hand and de Boer[12,13] on the other. Regarding the action upon the heart Jackson[14] published extremely interesting observations. As far as the general toxicity is concerned[4] there is a definite combination and alternation of both central stimulation culminating in convulsions, and depression with motor paralysis. Following preliminary observations made in collaboration with E. Käer, it was found that among the signs of stimulation the most consistent action is the ejaculation induced by the drug in male guinea pigs.†

Procedure. Mature male guinea pigs varying in weight between about 500 and 900 g were kept on a mixed diet. After some days of acclimatisation they were used for the experiments. Subcutaneous injections with 1% Gravitol solution were made in 7 different doses, from 10 to 50 mg per

* Partially aided by The Rockefeller Foundation.

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3 Barkan, G., *Arch. f. exp. Path. u. Pharmakol.*, 1932, **167**, 84.

4 Käer, E., and Barkan, G., *Arch. f. exp. Path. u. Pharmakol.*, 1933, **170**, 111.

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6 Kingisepp, G., *Arch. f. exp. Path. u. Pharmakol.*, 1935, **177**, 587.

7 Anan, Shinji, *Fol. jap. pharmacol.*, 1929, **9**, 53.

8 Okazaki, Tadashi, *Jap. J. med. Sci.*, IV. Pharmacol., 1932, **6**, 23.

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10 Bijlsma, U. G., and van Dongen, K., *Arch. internat. Pharmacodynamie*, 1937, **55**, 257.

11 Bijlsma, U. G., and van Dongen, K., *Arch. internat. Pharmacodynamie*, 1937, **55**, 265.

12 de Boer, S., *Arch. internat. Pharmacodynamie*, 1936, **54**, 65.

13 de Boer, S., *Arch. internat. Pharmacodynamie*, 1937, **55**, 262.

14 Jackson, D. E., *Experimental Pharmacology and Materia Medica*, 2d Ed., 1939, St. Louis.

† A preliminary report has been presented before The American Society for Pharmacology and Experimental Therapeutics, Boston Meeting, April, 1942.

kg of body weight. From previous experiments[4] the last dose was known to be lethal in about 50% of the animals. Between 6 and 13 animals were used in each group of dosage.

Results. Ten mg of Gravitol per kg of body weight were entirely ineffective. With larger doses an increased percentage of the animals showed ejaculation. In almost all of the experiments with ejaculatory response living spermatozoa were found, and there was also the typical coagulation of the ejaculate *in situ,* caused by the known action of the prostatic enzyme vesiculase.[15] Only in a very few instances one of the two phenomena was missing, *i.e.,* there were spermatozoa present but no coagulation, or coagulation occurred but no spermatozoa were found under the microscope. Table I shows the results obtained in 59 experiments, 37 of which were positive in the named respect.

TABLE I.
Frequency of Ejaculatory Response of Male Guinea Pigs with Varying Subcutaneous Doses of Gravitol.

Gravitol subc. mg per kg	10	20	25	30	35	40	50
No. of animals tested	6	7	6	7	13	13	7
No. of animals responding	0	1	2	5	10	12	7
Frequency of ejaculation, %	0	14.3	33.3	71.4	76.9	92.4	100

Applying Kärber's[16] method of mathematical treatment to the experimental results, 27.9 mg per kg of body weight were calculated as the dose which would show an ejaculatory response in 50% of the animals. Plotting the dose, or the log of the dose, against the frequency of positive effect typical S-curves are obtained. By graphical interpolation, either on these S-curves, or on the straight line obtained in substituting the percentage of positive response by the normal equivalent deviation,[17] between 27 and 28 mg of Gravitol per kg of body weight was found to be the 50% effective dose in guinea pigs, in good agreement with the above calculated value.

Table I (and the curve constructed from the tabulated data) shows definitely that the ejaculatory effect of the drug is a specific one. In all instances the ejaculation is intravital, occurring within 10 min to one hr after injection. Only once the effect was noticed after 7 min and once after more than one hr (66 min). Incidentally in both instances the dose of 40 mg per kg body weight had been injected. This in accordance with other observations indicates that the latent period between injection of the drug and ejaculation is not dependent upon dosage. In many experiments, particularly with larger doses, repeated ejaculations were observed. With 40 mg per kg more than 90% of the animals recover within 1 to 2 hr (only one lethal effect out of 13 cases). After a few days' rest the guinea pigs can be used again.

Utilizing this fact the consistency of the sensitivity in the same guinea pig on subsequent administrations was tested in a series of experiments. The examples listed in Table II would show that the individual sensitivity to the drug with respect to the ejaculatory response, though varying from animal to animal, is rather consistent in the same animal.

TABLE II.
Ejaculatory Response of the Same Guinea Pig to Repeated Administration of Gravitol.
(Doses per kg body weight)

Guinea pig No.	First experiment	Second experiment	Third experiment
142	25 mg positive	25 mg positive	25 mg positive
147	40 mg positive	35 mg positive	25 mg negative
149	40 mg negative	35 mg negative	50 mg positive

It might be mentioned that identical effects could be brought about by intragastric administration of tenfold doses; 200 mg per kg was the lowest dose which on gastric application caused ejaculation.

A few informatory experiments were car-

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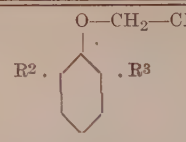
16 Kärber, G., *Arch. f. exp. Path. u. Pharmacol.,* 1931, **162,** 480.

17 Hemmingsen, A. M., *Quart. J. Pharm. Pharmacol.,* 1933, **6,** 39.

TABLE III.
Positive and Negative Ejaculatory Response of Guinea Pigs to Gravitol Homologs.



	Positive.				
R^1:	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_3H_5
R^2:	C_3H_5 (allyl)	$CH_3CH{=}CH$	C_3H_5	C_4H_7	OCH_3
R^3:	OCH_3 Gravitol	OCH_3	OC_2H_5	OCH_3	OCH_3
	Negative.				
R^1:	C_2H_5	C_3H_5	C_3H_5	C_3H_5	C_3H_5
R^2:	C_3H_5	C_4H_7	C_3H_5	$COOCH_3$	C_3H_5
R^3:	$COOCH_3$	OCH_3	—	OCH_3	OC_2H_5

ried out with some homologs of Gravitol. The results shown in Table III are qualitatively grouped as positive and negative depending on whether or not there was any ejaculatory response to subcutaneous injection of the respective hydrochlorides in any dosage in guinea pigs. None of the derivatives listed as positive reached the effectiveness of the original drug. The experience in this respect, however, is definitely incomplete and of purely preliminary nature. Yet, even with this limitation, the synopsis might be of some value for further study concerning the dependence of ejaculatory effect on chemical structure.

Discussion. Although in most of the positively reacting cases convulsions did occur, the convulsions obviously are not the dominating but rather a coördinated effect in the ejaculatory response to Gravitol. The reasons for this conclusion are:

(1) There were instances, particularly with the smaller doses of Gravitol, in which convulsions did not develop but only signs of minor central stimulation, and yet the ejaculatory response occurred.

(2) In other cases in which convulsions did develop later, the ejaculation occurred prior to the convulsions.

(3) With a superficial ether-anesthesia convulsions could be avoided or suppressed without abolishing the ejaculatory effect. Also with a barbiturate the same was found to be true; 30 to 40 mg of sodium amytal per kg guinea pig given subcutaneously produced anesthesia and sleep for almost 3 hr. Gravitol when injected in doses of 40 mg per kg during full anesthesia (½ to 1 hr after the injection of the hypnotic) was ineffective. The same guinea pigs, however, a few days later, when given even a smaller dose of Gravitol (30 mg per kg), but 7 to 10 min after sodium amytal, reacted while narcotized with typical ejaculation without convulsion or motor stimulation.

A few years ago Loewe[18–20] demonstrated that certain combinations of hypnotics and stimulants will cause prompt ejaculation in albino mice. It is interesting, as may be stated again, that the drug here studied shows both depressing and stimulating actions on the central nervous system.[4] It seems probable that these two actions incidentally combined in one single drug are responsible for the ejaculatory effect. The site of central stimulation causing the ejaculatory reflex is most likely identical with the one in the ejaculation produced by electric stimulation of the brain of the lightly anesthetized guinea pig.[21–23]

Summary. 1. The di-ethyl-amino-ethyl-ether of 2-methoxy-6-allyl-phenol in form of its hydrochloride (Gravitol, Clavitol, Uterol) was found to elicit a characteristic ejaculatory reflex in the mature male guinea pig.

2. This characteristic effect is attributed to the previously studied combination of the drug's depressing and stimulating action upon the central nervous system.

18 Loewe, S., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 483.

19 Loewe, S., *J. Pharmacol. and Exp. Therap.*, 1938, **63**, 70.

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21 Batelli, F., *C. R. Soc. de physique et d'histoire naturelle de Geneve*, 1922, **39**, 73.

22 Moore, C. R., and Gallagher, T. F., *Am. J. Physiol.*, 1929, **89**, 388.

23 Moore, C. R., and Gallagher, T. F., *Am. J. Anat.*, 1930, **45**, 39.

13824 P

Use of the Chick Embryo in Evaluating Disinfectants.*

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From the Department of Bacteriology, Ohio State University, Columbus.

The recognition of the limited value of the phenol coefficient test in evaluating wound disinfectants has led to a search for methods by which the tissue toxicity as well as the germicidal action of those agents can be determined *in vitro*. While suggested methods[1,2,3] differ in detail, they all measure the toxicity of disinfectants for tissues and for the test bacteria. If the disinfectant is less toxic for tissue than for the test organisms, it is inferred that the disinfectant is therapeutically active. However, such an interpretation may not be entirely justified, since the problem of wound disinfection is highly complex and the factors that influence the process are diverse. This being the case, efforts should be continued to develop a reliable, easily reproducible and inexpensive method for determining the actual therapeutic value of disinfectants *in vivo*.

In this report, experiments are decribed indicating the utility of the infected chorio-allantoic membrane of the developing chick embryo in studies on disinfectants. To illustrate the application of this method, results of testing the efficacy of aqueous solutions of phenol, iodine, and cetyl pyridinium chloride are presented.

Using the technic of Goodpasture and Buddingh,[4] windows were cut in the shells of eggs containing 11-day-old embryos, and a cover slip was placed over the opening of each. On the following day the chorio-allantoic membranes were inoculated with 0.02 cc of a 1:10 dilution (about 1,500,000 organisms) of a 23-25 hr broth culture of *Staphylococcus aureus* (F.D.A. strain 209). Eighteen hours later and on each of the 5 days following, 0.2 cc of the test disinfectant was dropped on the membrane. On the day after

TABLE I.
Therapeutic Effect of Disinfectants on Staphylococcal Infections of the Chorio-allantoic Membrane.

Disinfectant in aqueous solution	Dilutions tested	No. embryos infected	No. surviving infection and treatment	No. survivors showing degree of infection			
				0	+	++	+++
Phenol	1:100	17	14	0	0	0	14
	1:500	16	12	0	0	0	12
	1:1,000	13	8	0	0	0	8
Iodine	1:2,500	19	4	0	0	1	3
	1:5,000	25	16	0	0	0	16
	1:10,000	20	10	0	0	0	10
Cetyl pyridinium chloride	1:2,500	18	7	1	1	3	2
	1:5,000	24	17	0	1	4	3
	1:10,000	13	10	1	1	3	5
	1:20,000	12	10	0	0	1	9
Controls—Saline		30	20	0	0	0	20

* This investigation was supported by a grant from the William S. Merrell Co., Cincinnati, Ohio.

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2 Welch, Henry, and Hunter, A. C., *Am. J. Public Health*, 1940, **30**, 129.

3 Bronfenbrenner, J., Hershey, A. D., and Doubly, J. *J. Bact.*, 1939, **37**, 583.

4 Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hygiene*, 1935, **21**, 319.

the sixth treatment, the membrane was stroked with a moist cotton swab and with it half of a nutrient agar plate was streaked. The number of bacteria recovered on the petri plate was considered to be an indication of the degree of infection and was recorded as follows: 0 = no colonies; + = 1-10 colonies; ++ = more than 10, but plate not covered; +++ = plate covered with growth.

The results shown in Table I indicate that the infected chorio-allantoic membrane of the developing chick embryo may be used to demonstrate definite and decisive differences in the therapeutic value of disinfectants. These data represent the results of several experiments and since the results of such tests were similar, one may conclude that the findings are readily reproducible. The method as described makes it possible to determine with accuracy the comparative value of disinfectants under conditions more comparable to infected wounds than can be obtained by any *in vitro* method now available.

Insofar as the disinfectants themselves are concerned, it is evident from the data reported that (1) phenol did not show any demonstrable beneficial action, (2) with the exception of one embryo, iodine had no therapeutic effect, and (3) cetyl pyridinium chloride had a definite curative effect on the experimental infection.

13825

p-Aminobenzoic Acid and Sulfonamides in Rat Nutrition.

Gustav J. Martin. (Introduced by Walter Goldfarb.)

From the Warner Institute for Therapeutic Research, New York City.

Nutritional experimentation involving p-aminobenzoic acid[1-5] and the sulfonamides[6,7,11,12,13] has disclosed a similarity of action of the two agents. The role of both sulfonamides[10] and p-aminobenzoic acid[5] in altering the microflora of the intestine suggested the investigation under consideration in this report.

Three series of black rats were placed on diets with and without p-aminobenzoic acid and sulfanilamide.

Basal Diet.	
Vitamin free casein	18
Sucrose	67
Salts	4
Butter fat	9
Cod liver oil	2

Vitamin Supplements per kg of Diet.	mg
Thiamine hydrochloride	5
Riboflavin	10
Pyridoxine	5
Nicotinic acid	100
Calcium pantothenate	100
Choline chloride	200
Inositol	200

Series 1: 20 black rats, weighing 40 to 50 g, were placed on the above diet containing the 7 B complex vitamins without p-aminobenzoic acid or sulfanilamide. On this diet, as previously reported,[5] rats develop symptoms which terminate in death in 6 or 7

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2 Martin, G. J., and Ansbacher, S., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 118.

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5 Martin, G. J., *Am. J. Physiol.*, 1942, **136**, 124.

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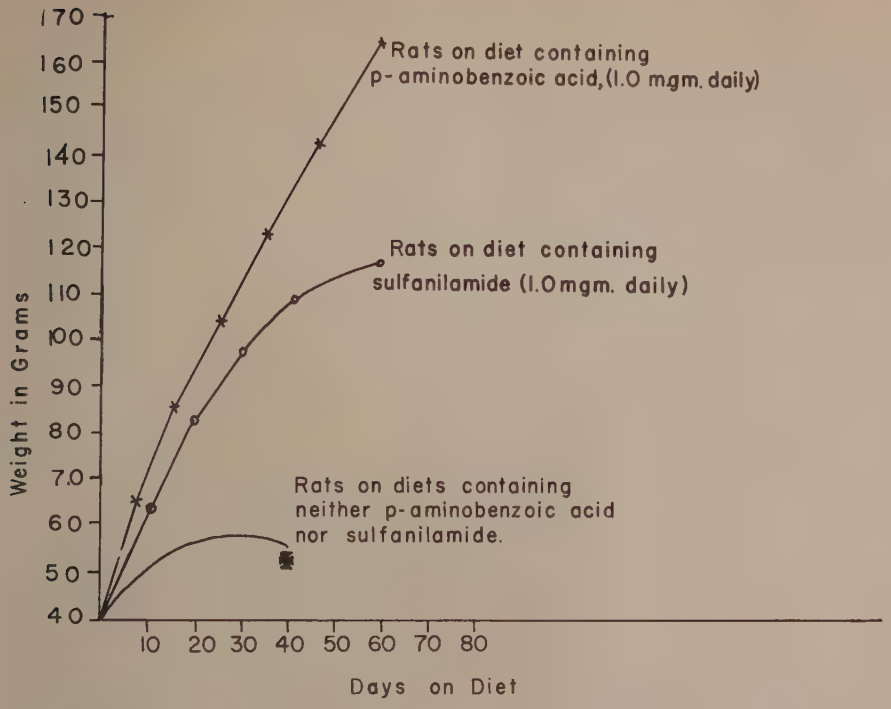
12 Daft, F. S., Ashburn, L. L., Spicer, S. S., and Sebrell, W. H., *U. S. Pub. Health Reports*, 1942, **57**, 217.

13 Welch, A. D., *Fed. Proc.*, 1942, **1**, 171.



THE EFFECT ON GROWTH OF RATS OF p-AMINOBENZOIC ACID AND SULFANILAMIDE IN SYNTHETIC DIETS.

weeks. The weights attained vary from 50 to 70 g. (Fig. 1).

Series 2: 20 black rats, weighing 40 to 50 g, were placed on a diet containing 7 of the vitamins listed, plus p-aminobenzoic acid (1 mg daily). These rats, as previously reported,[5] seem normal in every respect except that the coats are brownish with a slight residual greying. The weights at 6 weeks range from 110 to 180 g. (Fig. 1). These animals, after prolonged periods on the diet (6 months to a year) develop an appearance suggesting hyperthyroidism. The eyes seem to protrude, the white of the eye being more than usually visible. It might be that this effect is produced by abnormal withdrawal of the eyelid, as it was found that these animals[8] actually possess an atrophied thyroid and not a hypertrophied thyroid.

Series 3: 20 black rats, weighing 40 to 50 g, were placed on the synthetic diet containing 7 of the B complex factors and sulfanilamide (1 mg daily). At 6 weeks, these animals vary in weight from 90 to 160 g. They show coal black coats, and none of the brownishness or the slight residual greying seen in series No. 2 is evident in this group. At 10 weeks, the growth curves (Fig. 1) plateau, and some of the rats begin to lose weight gradually.

The experiments[5] demonstrating the action of p-aminobenzoic acid in precipitating a syndrome prevented by inositol suggested the possibility that the syndrome associated with or produced by the inclusion of sulfonamides[7,11,12,13] in synthetic diets was due to a similar action. Examination of the diets used by these investigators[7,11,12,13] revealed the absence of inositol from any of the synthetic diets fed, and it seemed logical, therefore, to repeat these experiments and to extend them by adding inositol to the diet of one series.

[8] Hueper, W. C., and Martin, G. J., unpublished data.

Series 4: 10 black rats (40 to 50 g in weight) were placed on the basal diet as described but receiving supplements of thiamine, riboflavin, pyridoxine, nicotinic acid, calcium pantothenate and choline chloride. If sulfanilamide or sulfaguanidine was added to this diet at 0.5%, the rats failed to grow and developed the characteristic[5] syndrome of an inositol deficiency.

Series 5: 10 black rats (40 to 50 g in weight) were placed on the basal diet containing 8 B complex factors (the six listed above plus p-aminobenzoic acid and inositol), with sulfanilamide or sulfaguanidine present at 0.5%. Rats on this diet did not develop the syndrome associated with an inositol deficiency,[5] but they did plateau in 60 days at weights markedly lower than those of Series 6. They develop a syndrome in which bleeding of the eyes, progressing to a severe panophthalmitis, is the most prominent symptom.

Series 6: 10 black rats (40 to 50 g in weight) were placed on our stock ration to which sulfaguanidine (2%) or sulfanilamide (1%) had been added. These animals grew normally and were normal in all macroscopic aspects.

In order to circumvent the complications inherent in the use of both sulfonamides and p-aminobenzoic acid in the same diet, the use of a different type of intestinal antiseptic seemed indicated. Aseptoform, the methyl ester of p-hydroxybenzoic acid, was selected because of its low toxicity.

Series 7: 10 black rats (40 to 50 g in weight) were placed on our stock diet supplemented by 2% aseptoform. Here again, as in the case of the sulfonamide stock diet, the animals grew normally and seemed normal in every respect.

Series 8: 10 black rats (40 to 50 g in weight) were placed on the synthetic diet previously described containing 8 B complex factors but with 2% aseptoform added. Animals on this diet die in 4 to 6 weeks, having attained weights of from 40 to 70 g. The most prominent symptoms are those of bleeding from the eyes, which progresses to a severe pan-ophthalmitis and a swollen edematous head.

Series 9: 10 black rats (40 to 50 g in weight) were placed on the same synthetic ration as series 8, but the aseptoform comprised only 0.6% of the ration. This group did not differ significantly from group 8 in growth performance or in symptomatology.

Discussion. Sulfanilamide replaces p-aminobenzoic acid to a considerable extent in the diet of the rat. The growth curve of rats on the diet containing sulfanilamide but no p-aminobenzoic acid plateaued at 10 weeks, while that of the p-aminobenzoic acid fed rats did not. When the sulfanilamide dosage was increased 1000%, the same growth curve was observed. The two agents, sulfonamide[10] and p-aminobenzoic acid[5] produce a similar effect: namely, the inhibition of the growth of certain microörganisms. Using either compound as a dietary component, the syndrome of an inositol deficiency can be produced, and the syndrome associated with or resulting from the inclusion of inositol in the diet can be prevented by p-aminobenzoic acid or partially prevented by sulfanilamide.

Sulfonamides added to stock diets in percentages as high as 5% do not significantly alter the nutritional behavior of the animal. Sulfonamides added to synthetic diets precipitate a syndrome of severe deficiency. Black *et al.*[11] found that liver extract, and to a lesser degree p-aminobenzoic acid, prevented the growth inhibiting effect of sulfaguanidine in rats fed a purified ration. These results were independently confirmed by Mackenzie *et al.*[7] The investigators[7,11] did not eliminate inositol as a factor in their diets. Sulfaguanidine at 1% added to a synthetic diet was found by Daft *et al.*[12] to result in the development of hyaline sclerosis and calcification of blood vessels in rats on this diet. Both inositol and p-aminobenzoic acid were absent from the diet of these investigators. Welch[13] found no toxic manifestations and no depression of growth of rats when he added succinyl sulfathiazole at 5% to stock diets on which the rats were kept. Although p-aminobenzoic acid did not correct the syndrome developed when this sulfonamide was added to synthetic diets, liver did.

Our observations have demonstrated that

diets containing 8 B complex factors, including p-aminobenzoic acid and inositol, do not afford adequate nutrition if sulfonamides are included. This offers evidence for the contention[11] that another factor of the B complex is involved, particularly as liver extracts will correct this dietary inadequacy. The potentialities of toxicity of the sulfonamide in the diet led us to attempt to produce a similar condition using another intestinal antiseptic, aseptoform, the methyl ester of p-hydroxybenzoic acid. Stock diets containing 2% aseptoform were fed to rats and mice with no evidence of toxicity. Aseptoform added at 2% and at 0.6% to synthetic diets containing 8 B complex factors resulted in the development of the characteristic deficiency syndrome in rats on this diet. There being no interrelationship between p-aminobenzoic acid and aseptoform, this type of diet seemed superior to a sulfonamide and p-aminobenzoic acid-containing diet. The syndrome in rats produced by the inclusion of either aseptoform or sulfonamide in the diet is essentially the same. It seems, therefore, improbable that the factor of toxicity enters the picture. It is suggested that another factor exists which is synthesized by the bacteria of the gastro-intestinal tract when rats are placed on synthetic diets, or which is present in stock diets of natural foods, with the result that rats on these diets supplemented by sulfonamide or aseptoform do not develop a deficiency.

Summary. Sulfanilamide, replacing p-aminobenzoic acid in a synthetic diet containing 7 B complex factors including inositol, prevents the development of the syndrome heretofore associated with a deficiency of p-aminobenzoic acid, but it does not entirely replace p-aminobenzoic acid.

Intestinal antiseptics (sulfonamides and aseptoform) added to synthetic diets containing 8 B complex factors make possible the demonstration in rats of a deficiency syndrome which it is suggested may be due to a ninth B complex factor.*

* Preliminary results indicate biotin is not the factor.

13826

Nicotinic Acid in Chick Nutrition.*

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The activity of nicotinic acid in the prevention and cure of canine blacktongue and human pellagra is well established. There has been much speculation, however, as to the importance of this vitamin in the diet of the chick and rat.

Mickelsen, Waisman, and Elvehjem[1] and Dann and Subbarow[2] have demonstrated that nicotinic acid is inactive in preventing or curing chick dermatitis, previously described as chick pellagra by Ringrose, Norris and Heuser.[3] This antidermatitis factor was later shown to be pantothenic acid by Wool-

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We are indebted to Merck and Company, Rahway, N.J., for the crystalline vitamins; to Wilson Laboratories, Chicago, Ill., for solubilized liver and gelatin; and to Allied Mills, Peoria, Ill., for soy bean oil.

1 Mickelsen, O., Waisman, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1938, **124**, 313.

2 Dann, W. J., and Subbarow, Y., *J. Nutr.*, 1938, **16**, 183.

3 Ringrose, A. T., Norris, L. C., and Heuser, G. F., *Poul. Sci.*, 1931, **10**, 166.

4 Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1939, **129**, 673.

ley, Waisman, and Elvehjem[4] and by Jukes.[5] In a more recent paper Waisman and Elvehjem[6] reported that variable results with chicks were obtained when nicotinic acid was added to a modified Goldberger ration, growth responses being noticed in several groups. The authors concluded, however, that the ration did not lend itself for the demonstration that the chick requires this vitamin.

In our work with the chick, using purified rations, we had occasionally noted small growth responses due to the addition of nicotinic acid. Upon further study we have been able to construct a ration fairly complete in all the necessary factors and yet sufficiently low in nicotinic acid to permit the study of the role of this vitamin in chick nutrition. We have observed in the absence of nicotinic acid a depression in the growth rate and the appearance of a deficiency condition similar to canine blacktongue.

The basal ration, 486N, had the following composition per 100 g: dextrin 61 g, alcohol extracted casein 18 g, gelatin 10 g, salts IV[7] 5 g, Ca HPO_4 1 g, soy bean oil 5 g, eluate fraction $\cong$ 5 g of solubilized liver,[8] *l*-cystine 300 mg, thiamine HCl 300 γ, riboflavin 600 γ, biotin concentrate (S.M.A. No. 1000) 15 γ, pyridoxin HCl 400 γ, Ca pantothenate 1.5 mg, choline Cl 150 mg, *i*-inositol 100 mg, 2-methyl-1, 4-naphthoquinone 500 γ, and α-tocopherol 300 γ. Each chick received 3 drops of a vitamin A and D concentrate weekly. A microbiological assay of this basal ration† showed a nicotinic acid content of 0.3 mg per 100 g of ration.

Day-old white Leghorn chicks were divided into groups of 6 and placed in electrically heated cages with raised screen bottoms. The experimental rations, prepared weekly, and water were supplied *ad libitum*. Crystalline nicotinic acid (Merck) was used throughout.

TABLE I.
Effect of Supplementary Nicotinic Acid on Growth of Chicks.

Supplement to basal ration (486N) Mg nicotinic acid per 100 g	No. of chicks	No. dead	Wt at 3 wks g	Wt at 4 wks g
No supplement	18	1	106	146
0.5	6	0	123	
1.0	6	0	153	
1.5	6	1	164	
2.0	6	0	163	
2.5	11	0	164	
5.0	6	0	173	
10.0	18	0	163	226

The results are summarized in Table I. On the basal ration the chicks grew at a relatively slow rate, weighing 106 g at 3 weeks of age. With chicks receiving higher levels of nicotinic acid the growth rate correspondingly increased until a level of 1.5 mg per 100 g of ration was reached. These chicks weighed 164 g at 3 weeks, 58 g better than the negative control groups. Higher levels did not appreciably increase the growth rate. Therefore, since the basal ration contained 0.3 mg of nicotinic acid per 100 g, the minimal level required was approximately 1.8 mg per 100 g of ration.

Beginning at about 2 weeks of age the entire mouth cavity of the deficient chicks, as well as the upper portion of the esophagus and the crop, became distinctly inflamed with a deep red color; however, the tip of the tongue appeared nearly white. The mouths of the control chicks, in contrast, remained a normal pink. A decrease in food consumption was also noticed. Upon the addition of nicotinic acid to the ration, food consumption and growth increased and the chick "blacktongue" symptoms disappeared within a few days. A lower supplementary level of nicotinic acid (0.5 mg per 100 g of ration) was required for the prevention of "blacktongue" symptoms than for maximal growth. Nicotinic acid amide had the same activity as nicotinic acid in curing deficiency symptoms.

5 Jukes, T. H., *J. Am. Chem. Soc.*, 1939, **61**, 975.

6 Waisman, H. A., and Elvehjem, C. A., *J. Nutr.*, 1940, **20**, 519.

7 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

8 Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521; Mills, R. C., Briggs, G. M., Jr., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 186.

† We wish to thank Mr. L. J. Teply for this assay.

Dann and Handler[9] and Snell and Quarles[10] have given evidence for the occurrence of a marked increase in the nicotinic acid content of the incubating egg and have suggested, therefore, that the hatched chick does not require a dietary source of this vitamin. From our experiments we may conclude that if such synthesis of nicotinic acid does exist in the growing chick there is not sufficient formation to supply enough of the vitamin for optimal growth and for the prevention of chick "blacktongue." The question of the synthesis of nicotinic acid by the chick can only be answered by further experimentation.

Summary. The growing chick requires a dietary source of nicotinic acid for optimal growth, and for the prevention of chick "blacktongue" as shown by the use of highly purified rations. The minimal level of this vitamin needed is approximately 1.8 mg per 100 g of ration.

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10 Snell, E. E., and Quarles, E., *J. Nutr.*, 1941, **22**, 483.

13827

Effect of Liver Damage on Urinary Morphine Excretion.

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Gross, Plant and Thompson[1] reported that liver damage by chloroform caused an increased urinary excretion of morphine in both tolerant and non-tolerant dogs. At the time of this report the presence of conjugated morphine was not recognized. Gross and Thompson[2] demonstrated that dogs excreted morphine in a combined form, and Oberst[3] in the same year showed that man also excreted a combined form of morphine. The excretion of combined morphine has shown that morphine undergoes far less destruction in the animal body than formerly supposed. While the nature of the combined morphine excretion product is still unknown, Oberst[4] believes that the morphine is conjugated with glucuronic acid. He has further demonstrated that the conjugation occurs with the hydroxyl groups and that both the phenolic and alcoholic hydroxyls may be conjugated.

Thompson and Gross[5] studying the combined morphine excretion compound in dog urine found that in this animal the combined morphine is excreted in two forms, namely a fraction which is easily hydrolyzed and the other which hydrolyzes only with more drastic procedure.

With the information that liver damage caused an increase in the excretion of free morphine in dogs, a study of the effect of liver damage on the combined morphine was undertaken in both tolerant and non-tolerant dogs.

Five non-tolerant and 3 tolerant animals were used in these experiments. The dogs were maintained on a constant diet and water intake. Morphine determinations were made before and after liver damage on a constant dose of morphine. As an indication of hepatic damage bromsulphalein dye determinations were also made before and after the liver damage. Five mg per kg of the dye were injected intravenously and the dye in 1 cc of clear serum determined at 3, 8 and 15 min after the injection. Carbon tetra-

1 Gross, E. G., Plant, O. H., and Thompson, V., *J. Pharm. and Exp. Therap.*, 1938, **63**, 13.

2 Gross, E. G., and Thompson, V., *J. Pharm. and Exp. Therap.*, 1940, **68**, 413.

3 Oberst, F. W., *J. Pharm. and Exp. Therap.*, 1940, **69**, 240.

4 Oberst, F. W., *J. Pharm. and Exp. Therap.*, 1941, **73**, 401.

5 Thompson, V., and Gross, E. G., *J. Pharm. and Exp. Therap.*, 1941, **72**, 138.

TABLE I.
Non-tolerant Female. Weight 5 kg.

Days	Mg morphine inj. daily	Urine vol. 24 hr	Mg morphine recovered				Bromsulphalein in serum Min after injection		
			Total	Free	Easily Hydrolyzed	Difficultly Hydrolyzed	3	8	15
1	100	172	68	11.2	3.2	53.6			
2	100	209	73	12.6	5.1	55.3			
3	100	250	76	11.9	4.9	59.2			
4	100	190	78	12.1	4.8	60.6	.036	.015	.00
Avg			73.7	11.9	4.5	57.2			
(5-12)	None								
13	100	350	82	15.9	2.6	63.5			*
14	100	165	69.5	23.2	1.3	54.5	.036	.02	.006*
15	100	385	87	34.4	0.0	52.6			
16	100	180	86.3	32.0	1.5	52.8	.038	.025	.012
17	100	168	79.1	27.0	1.2	50.9			
Avg			80.8	26.5	1.32	54.8			

*15 cc CCl_4 in 200 cc H_2O.

TABLE II.
Tolerant Female. Weight 5.5 kg.

Days	Mg morphine inj. daily	Urine vol. 24 hr	Mg morphine recovered				Bromsulphalein in serum Min after injection		
			Total	Free	Easily Hydrolyzed	Difficultly Hydrolyzed	3	8	15
1	110	325	39.9	8.3	9.0	22.6	Control period		
2	110	375	46.6	12.4	10.6	23.6			
3	110	250	46.0	10.3	8.0	27.7			
4	110	200	42.8	11.8	8.9	22.1	.032	.01	.00
Avg			43.8	10.7	9.1	24.0			
5	110	275	48	11.5	7.1	29.4			*
6	110	330	56	15.0	2.0	39.0	.036	.02	.004*
7	110	365	42.5	15.8	3.3	23.4			
8	110	240	42.8	27.0	4.0	11.8			†
9	110	400	46.2	13.2	2.0	30.7			
10	110	200	45.7	26	.00	29.7	.05	.04	.025
11	110	220	48.2	23.0	.00	25.2			
12	110	190	43.6	20.9	.00	22.7			
13	110	280	40	17.3	.00	23.7	.05	.036	.034
14	110	230	36	15.5	1.7	18.8			
15	110	210	48.5	17.3	1.5	29.7			
Avg			45.2	18.4	1.9	25.8			

*11 cc CCl_4 in 100 cc water.
†27.5 cc CCl_4 in 200 cc water.

chloride was used as the liver damaging agent. The agent was administered orally in doses of 2-5 cc per kg body weight after fasting the animal for 24 hr. The carbon tetrachloride was given in repeated or single doses. The urine was analyzed for total, free and easily hydrolyzable morphine according to the procedure of Thompson and Gross.[5] The difficultly hydrolyzable morphine fraction is obtained by difference.

Results of such studies on a non-tolerant and a tolerant dog are given in Tables I and II.

From the results in Tables I and II, the delayed disappearance of the dye indicates some hepatic damage after the administration of carbon tetrachloride. Our earlier observation[1] that liver damage by chloroform

produced an increase in free morphine excretion, also holds true when carbon tetrachloride was used as the damaging agent. In addition to the marked increase in the free morphine there is a decrease in the excretion of the easily hydrolyzable fraction of the combined morphine. As the total recoverable morphine is not materially altered and the difficultly hydrolyzable portion remains fairly constant, the easily hydrolyzed compound appears as free morphine.

One might interpret from these experiments that the easily hydrolyzed form is the only form of conjugation that takes place in the liver, and the formation of the difficultly hydrolyzable fraction occurs at other sites. However, until the conjugation products are identified and their metabolism studied, it is impossible to make any definite statements as to the site of the morphine conjugation. However, liver damage does inhibit the conjugation of morphine to a certain extent as shown by the fact that the free morphine found in the urine is increased without increasing the total morphine excreted.

13828

Relation of Pancreatic Secretion to Fatty Changes in the Liver.*

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The question whether following ligation of all pancreatic ducts infiltration of the liver can be prevented by pancreatic juice or by an internal secretion of the pancreas is still controversial.

Dragstedt and collaborators did not observe fatty livers following ligation of all pancreatic ducts, nor following complete loss of all external secretion of the pancreas, and they could not prevent or cure fatty livers by administration of large amounts of pancreatic juice.[1,2] This has been in part confirmed by Boyce and McFetridge,[3] but the results of a number of investigators have been opposed to their findings.[4–10] The latter group found fatty livers following ligation of all pancreatic ducts and Montgomery and collaborators were able to prevent these changes by the feeding of pancreatic juice.[9,10] Our observations in long term experiments on dogs may contribute to decide this controversy.

Results. Two animals, with all pancreatic ducts ligated and cut in an aseptic operation and with omentum interposed between duodenum and pancreas were kept for about one year. Both dogs had a good appetite and were kept on a ratio of bread, vegetables, meat and a yeast-bonemeal-salt mixture, with weekly feedings of cod liver oil and dried ox blood. One of the animals lost weight

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1 Van Prohaska, J., Dragstedt, L. R., and Harms, H. P., *Am. J. Physiol.*, 1936, **117**, 166.

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10 Montgomery, M. L., Entenman, C., Chaikoff, I. L., and Nelson, C., *J. Biol. Chem.*, 1941, **137**, 693.

11 Popper, H. L., and Sorter, H. H., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 384.

slowly and continually, while the weight of the other one did not change materially. Fasting blood sugars were normal. The curves for serum amylase and lipase showed the usual course.[11] Both dogs died 11½ and 12½ months respectively after operation, one with acute enteritis and the other one from trauma and hemorrhage due to a swallowed stone. In both dogs autopsy showed that the pancreas was completely atrophic, appearing as a small, thin, cordlike mass. On microscopic examination it contained scar tissue, many nerve cells and capillaries, an abundance of islets of Langerhans, and very small nests of necrotic acinar tissue. The liver was enlarged and yellow, and on microscopic examination such an extreme degree of fatty infiltration was seen that the characteristic structure was almost obscured.

In 2 other dogs, only the main pancreatic duct was ligated and severed in an aseptic operation and the animals were kept for 8½ and 10½ months respectively on the diet described above. One of these dogs did not lose weight, but the other one showed a marked continuous loss of weight starting 3 months after operation. Autopsy of both dogs showed a pancreas which was completely atrophied with the exception of a piece of normal pancreatic tissue around the small accessory duct, measuring 3 cm in length and 3 mm in width in one dog and being of the size of a peanut in the other dog. Thus only a small portion of the glandular pancreatic tissue had remained functioning. The liver of both these dogs was normal macroscopically and microscopically, with no abnormal fat deposits. The above findings of only a small area of intact pancreatic tissue was exceptional, because in a great number of dogs in whom a similar operation had been performed, much larger parts of the pancreas were found intact after prolonged periods of time.

Discussions and Conclusions. Our findings on the appearance of extreme fatty infiltration of the liver in dogs on a mixed diet following severance of all pancreatic ducts contradicts those of Dragstedt and collaborators,[1,2] and of Boyce and McFetridge,[3] and is in accord with those of Berg and Zucker,[4] Aubertin and collaborators,[5] Ralli and collaborators,[6] Person and Glenn,[7] and of Montgomery and collaborators.[8–10]

The last two of our experiments tend to demonstrate that it is the external secretion of the pancreas which, even in small quantities, can prevent the fatty infiltration of the liver cells. While our results confirm those of the second group of investigators mentioned above,[4–10] they do not explain such opposed findings from two groups of reliable workers. We feel that differences in the diets of the experimental animals may be of great importance in the appearance or in the prevention of fatty liver following ligation of all pancreatic ducts. This belief is based on recent findings on the importance of dietary factors, such as proteins, fat and carbohydrate content and balance,[12,13] vitamin B_1 content,[14,15] a balance between certain amino-acids,[16–19] a balance between certain proteins and substances with labile methyl

12 Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1935, **83**, 255.

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18 Singal, S. A., and Eckstein, H. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 512.

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21 Best, C. H., and Ridout, J. H., *Am. J. Physiol.*, 1938, **122**, 67.

22 Best, C. H., Grant, R., and Ridout, J. H., *J. Physiol.*, 1936, **86**, 337.

23 Florentin, P., and Grognot, P., *C. Rend. Soc. Biol.*, 1939, **130**, 348.

24 McHenry, E. W., and Gavin, G., *Science*, 1940, **91**, 171.

25 Ralli, E. P., and Rubin, S. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 601.

26 Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, 1941, **138**, 477.

groups[20,21] and on other nutritional factors not well known as yet.[22–26]

Summary. Two dogs with occlusion of all pancreatic ducts showed extensive fatty changes of the liver about 12 months after operation. Two dogs with occlusion of the main pancreatic duct in which only a very small fraction of the pancreas had remained functioning showed no fatty changes of the liver after 7½ and 8½ months respectively. The development of fatty liver is evidently not due to a pancreatic hormone but due to the lack of the external pancreatic secretion and a very small fraction of the normal pancreas and its secretion seems to be sufficient to prevent it.

13829

Bacterial Assimilation of Acetic and Succinic Acids Containing Heavy Carbon by *Aerobacter indologenes.**

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Slade *et al.*[1] have shown the fixation of $C^{13}O_2$ in the carboxyl group of acetic acid by *Aerobacter indologenes* and *Clostridium welchii*. The present report provides evidence for the occurrence of the reaction by a cleavage of a C_4-dicarboxylic acid. Participation of C_2-compounds in the synthesis of 2,3-butylene glycol by *Aerobacter indologenes* is also shown to occur.

Two types of acetic acid were synthesized, (1) $CH_3 \cdot C^{13}OOH$ and (2) $C^{13}H_3 \cdot C^{13}OOH$[2]. Succinic acid ($C^{13}OOH \cdot CH_2 \cdot CH_2 \cdot COOH$) was recovered from various bacterial fermentation. Salts of the acids were added to cell suspensions of *A. indologenes* in the presence of glucose under an atmosphere of nitrogen. The concentration of C^{13} was determined by mass spectrometer analysis. The normal complement of C^{13} is 1.09%.

The distribution of C^{13} on the addition of the acids is shown in Table I. Succinic acid formed in the presence of $CH_3 \cdot C^{13}OOH$ contains C^{13} exclusively in the carboxyl carbons (Table II). Succinate was not formed by a synthesis of pyruvate by C_1 and C_2 addition, followed by C_3 and C_1 addition to oxalacetate, for the succinate formed would have contained C^{13} in the methylene carbons. Thus, succinic acid is formed by means of a carbon to carbon linkage involving the carbon† atom originally present in the methyl group of acetic acid. The general reaction is:

$$\begin{matrix} CH_3 \cdot COOH \\ + \\ CH_3 \cdot COOH \end{matrix} \rightleftharpoons \begin{matrix} CH_2 \cdot COOH \\ \cdot \\ CH_2 \cdot COOH \end{matrix} + 2H$$

The reaction is presented with the reservation that acetic acid may or may not be the actual C_2 compound involved in the condensation reaction.

Succinate formed in the presence of $C^{13}H_3 \cdot C^{13}OOH$ contains C^{13} equally distributed between the methyl and carboxyl carbons (Table II). This is in agreement with the above reaction and proves that the acetate was not oxidized to $C^{13}O_2$ and the succinate formed by the Wood and Werkman reaction; otherwise the C^{13} would have been found exclusively in the carboxyl carbons.

The formation of C^{13}-acetate from succinate demonstrates that the condensation reaction is reversible. There is no mechanism known for the oxidation of succinate by which single carbon atoms may be split off leaving a C_2-residue containing a carboxyl carbon of the original succinate.

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1 Slade, H. D., Wood, H. G., Nier, A. O., Hemingway, Allan, and Werkman, C. H., *J. Biol. Chem.*, 1942, **143**, 133.

† Thanks are expressed to Dr. H. G. Wood for assistance in the preparation of this acid.

TABLE I.
Fermentation of Glucose Plus Organic Acids by *A. aerogenes.*

	Acid added	CO_2	Acetic acid	Succinic acid	Ethyl alcohol	2,3-Butylene glycol
$CH_3 \cdot C^{13}OOH$	2.39	1.08	2.01	1.21	1.64	1.21
$C^{13}H_3 \cdot C^{13}OOH$	4.51	1.12	3.27	1.33	2.19	1.24
$CH_2 \cdot C^{13}OOH$ · $CH_2 \cdot COOH$	1.57	1.09	1.22	1.37	1.18	1.15

Values expressed as per cent C^{13}.

TABLE II.
Location of Heavy Carbon of Assimilated Acetic Acid in Compounds Synthesized by *A. indologenes.*

	$CH_3 \cdot C^{13}OOH$ added	$C^{13}H_3 \cdot C^{13}OOH$ added
Succinic acid	1.21	1.33
methylene carbon	1.10	1.32
carboxyl carbon	1.25	1.29
2,3-Butylene glycol	1.21	1.24
methyl carbon	1.10	1.23
hydroxyl carbon	1.27	1.22
Ethyl alcohol	1.64	2.19
methyl carbon		2.16
hydroxyl carbon		2.17

The 2,3-butylene glycol formed in the presence of $CH_3 \cdot C^{13}OOH$ contains C^{13} exclusively in the hydroxyl carbons (Table II). Thus, a carbon to carbon linkage is created in the synthesis of the glycol which involves the carbon atom originally present in the carboxyl group of acetic acid. The glycol formed in the presence of $C^{13}H_3 \cdot C^{13}OOH$ contains C^{13} equally distributed between the methyl and hydroxyl carbons. Also the addition of C^{13} succinate results in the formation of glycol containing C^{13}. This result is comparable to those in which acetate was added because of the cleavage of succinate to acetate.

Hence, direct proof has been obtained for the participation of a C_2-compound, probably acetaldehyde, in the synthesis of 2, 3-butylene glycol by the intact cell of *Aerobacter.* It is possible that either (1) two molecules of acetaldehyde or (2) a molecule of acetaldehyde and a molecule of pyruvic acid are involved. The results of Green *et al.*[2] support the latter possibility.

Ethyl alcohol containing C^{13} is formed in the presence of acetic and succinic acids (Table I). On the addition of $C^{13}H_3 \cdot C^{13}OOH$, the alcohol formed contained C^{13} equally distributed between the methyl and carboxyl carbons, consequently a reduction of acetic acid to ethyl alcohol has occurred.

Approximate calculations indicate that the condensation of acetate to succinate and the reverse reaction, the condensation of acetate to 2, 3-butylene glycol, and the reduction of acetate to ethyl alcohol are reactions which possess quantitative significance under the experimental conditions.

2 Green, D. E., Westerfield, W. W., Vennesland, B., and Knox, W. E., personal communication.

13830

Comparison of Glycerol and Trehalose as Nutrients for the Growth of Tubercle Bacilli.*

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Glycerol is recognized as the most valuable organic nutrient for the growth of tubercle bacilli. The studies of Anderson,[1] however, show that the lipins which make up a large part of the structure formed by these bacilli in the course of growth are not glycerides, but in considerable measure are esters of trehalose. It therefore seemed of interest to see if trehalose might function more effectively than glycerol in promoting growth. Trehalose for the experiment was furnished by Dr. Anderson.

Two lots of Long's[2] synthetic medium were made up, one containing 5% of glycerol and the other 5% of trehalose instead of glycerol. Both lots were sterilized in the autoclave at 15 lb for 30 min. They were tested for reducing sugars after autoclaving and found negative. All flasks were inoculated with an equal amount of fresh tubercle bacillus culture (A-21), and incubated at 37.5°C for 58 days.

At the end of this time there was visibly less growth on the trehalose culture medium than on the glycerol medium. The total growth from each flask was filtered through tarred filter paper, air dried for several weeks, and weighed. The average dry weight of bacilli per 200 cc of the regular Long's medium containing glycerol was 1.580 g, and that from the same medium with trehalose substituted for glycerol was 0.985 g.

It thus does not appear that preformed trehalose has any advantages over glycerol as a constituent of the culture medium for tubercle bacilli.

* Aided by a grant from the Committee on Medical Research of the National Tuberculosis Association.

[1] Anderson, R. J., *Chem. Rev.*, 1941, **29**, 225.

[2] Long, E. R., and Seibert, F. B., *Am. Rev. Tuberc.*, 1926, **13**, 393.

13831

Effects of Intravenous Injections of L-Dopa upon Blood Pressure.*

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It has been reported by Bing and Zucker[1] that a rise in blood pressure is produced by the injection of l-dopa into the renal artery or directly into the renal parenchyma of cats after the kidneys of these animals have been made hypoxic by temporarily arresting the arterial blood supply. The rise in blood pressure has been attributed to a disturbance in the amino acid metabolism in the kidney. Normally in this organ aromatic amino acids are first decarboxylated to form amines with pressor properties, and the latter are then deaminized. Under anærobic conditions, although the decarboxylation is not affected, the deaminization does not take place (Holtz *et al.*).[2] As a result in the chronic ischemic kidney the pressor amines enter the blood

* This investigation was aided by a grant from the Winthrop Chemical Company.

† George Blumenthal, Jr., Fellow.

[1] Bing, R. J., and Zucker, M. B., *Am. J. Phys.*, 1941, **133**, P214.

[2] Holtz, P., Heise, C., and Luedtke, C., *Arch. Exp. Path. u. Pharm.*, 1938, **191**, 87.

stream and produce a rise in blood pressure. This absence of deaminization in chronic renal ischemia is also thought to be a factor in the production of animal experimental hypertension *per se*.

The work cited suggested that a similar mechanism might obtain in human essential hypertension, and a study of the mechanism in patients with this disease was, therefore, undertaken. Since injection directly into the renal artery in humans is highly impractical, it was first necessary to determine if peripheral intravenous injections‡ in hypertensive cats would produce the same effect as did the renal intra-arterial injections. The effect of l-dopa via intravenous injection was then studied in 4 normal cats, and again after acute renal ischemia was produced in the 4 cats by applying Goldblatt clamps to both renal arteries. Following this the right kidney was removed from each animal, the left renal artery clamp was left untouched. Three animals survived and developed hypertension. These animals were injected with l-dopa 10 days after operation, at which time their basal blood pressure readings averaged 40 mm above their original levels. The experiments were carried out while the animals were under nembutal anesthesia; the blood pressure was recorded by femoral artery cannulation.

Intravenous injection of 50 mg of l-dopa caused a marked rise, averaging 60 mm of mercury above the basal level, in the blood pressure of the hypertensive cats. The rise reached its maximum 4 min after the injection and persisted for a total of from 12 to 15 min. A second injection of l-dopa given after the maximum elevation had occurred caused no further increase in the blood pressure but prolonged the duration of the rise. The intravenous injection of 50 mg of l-dopa in the normal cats or cats with acute renal ischemia produced no rise in blood pressure. No untoward effects due to the dopa were observed in any of the cats.

Since these observations established the similarity in blood pressure response in hypertensive cats to peripheral intravenous and direct renal intra-arterial injections of l-dopa, a basis was given for a comparative study of the effect of intravenously administered l-dopa in human essential hypertension. Five patients with this malady and an equal number of patients with normal blood pressure were injected with the amino acid and the effect on the blood pressure was noted.

The hypertensive patients selected were individuals between the ages of 35 and 50 who had definite, but not excessive elevation of blood pressure. Hypertension was an incidental clinical finding in these patients. They presented no anamnestic or laboratory evidence of kidney damage, no signs or symptoms of cardiac decompensation, nor electrocardiographic changes indicative of myocardial damage. Each hypertensive patient was paired with a patient with normal blood pressure, both members of the pair receiving the same amount of l-dopa. The quantity injected varied between 100 and 450 mg dissolved in 10 to 30 cc of sterile physiological saline. In each experiment a quantity of saline equal to that used to dissolve the l-dopa was injected several minutes prior to the dopa injection to control the possible effect of the injection procedure itself on the blood pressure. The patients were kept in bed for at least several hours prior to, and also during the experiment. The blood pressure was measured at one minute intervals with the auscultatory method.

The injection of l-dopa in quantities of 120 mg or more produced a definite rise in the blood pressure of the hypertensive patients. The time of the onset after the injection, and the magnitude of the blood pressure rise bore a relationship to the amount of l-dopa injected. As may be seen in Table I, the larger the dose of the amino acid, the sooner the elevation in blood pressure occurred and the greater the magnitude of the elevation. No definite relationship could be ascertained between the amount of l-dopa used and the duration of the blood pressure increase.

The normotensive patient injected with 120 mg of l-dopa showed no elevation of blood pressure, although this amount of the

‡ A pressor response to intravenous injections of dopa in rats has been reported by Schroeder.[3]

[3] Schroeder, H. A., *J. Exp. Med.*, 1942, **75**, 523.

TABLE I.
Effect of Dopa Injection on Human Blood Pressure.

Pat.	Age	Basal B.P., mm Hg	Amt dopa inj., mg	Max. ht systolic pressure, mm Hg	Interval inj. to max. rise, min	Duration of rise, min	Max. rise of diastolic pressure, mm Hg	Side effect	Onset of side effect after inj., min
M.N.	49	134/74	80	0	—	0	0	0	—
V.A.	35	150/100	95	0	—	0	0	0	—
M.G.	45	110/66	120	0	—	0	0	0	—
S.M.	50	150/90	120	22	5.5	6	10	nausea, retching	4
E.S.	30	140/86	200	8	4.5	2	2	mild tachycardia	4
A.U.	42	174/90	200	30	4	6	6	mild tachycardia, tinnitus	2
G.S.	40	120/60	200	10	4	2.5	3	sweating, retching	4
R.T.	36	196/108	200	32	3	16	10	nausea	6
								vomiting	8
S.G.	39	112/70	450	18	2	13	8	sl. nausea	2
								vomiting	15
R.B.	41	170/105	450	35	1.5	15	15	nausea	4
								vomiting	7

amino acid had caused a rise in the hypertensive patient which lasted for 6 min and reached a maximum of 22 mm. Two normal patients injected with 200 mg of l-dopa exhibited rises of 8 and 10 as compared to 30 and 32 mm respectively in hypertensive patients who also received 200 mg of l-dopa. The duration of the rise in the normals was 2 and 2.5 min respectively, while that in the hypertensives was 6 and 16 min. The rise occurred slightly sooner in the hypertensives than in the normals. The injection of 450 mg in a normotensive patient produced a rise which lasted for 13 min and reached a maximum of 18 mm. In the hypertensive patient receiving the same dose there occurred a rise with a maximum of 35 mm and lasting 15 min. It will be noted that the injection in a hypertensive patient of 120 mg of dopa caused a higher rise than did the larger amounts including the 450 mg dose, in the patient with normal blood pressure.

Injection of 100 mg or less of l-dopa had no effect of the blood pressure of 2 patients with normal tension, and one patient with moderate hypertension. Two additional patients with marked hypertension, (B.P. 236/112 and 260/160), also showed no pressor response to small doses (75 and 100 mg) of l-dopa.

In addition to the pressor response side effects were observed in the patients injected with 120 mg or more of l-dopa. The hypertensive patient who received 120 mg experienced nausea and retching; the normotensive injected with this dose exhibited no side effects. One of the 2 patients with normal tension injected with 200 mg dopa had mild tachycardia, the other sweating and retching. Of the hypertensives receiving the same dose, one had mild tachycardia and tinnitus, the other nausea, vomiting and retching. Following the injection of 450 mg of dopa, both the normotensive and the hypertensive patient had nausea and vomiting. The vomiting caused no drop in blood pressure. In the majority of the cases, the side effects appeared after the maximum rise in blood pressure had developed. No other relationship of these side effects to the pressor effect could be established.

Discussion. Although the humans with essential hypertension received proportionally much smaller amounts of l-dopa than did the hypertensive cats, similar blood pressure responses were obtained in both. The similarity consisted in a rather abrupt definite rise in the blood pressure which persisted for several minutes and then returned to the basal hypertensive level. The normotensive patients also exhibited a pressor response, but the magnitude and duration of the blood pressure elevation was considerably less than that of the hypertensives receiving the same amount of l-dopa. The similarity of the l-dopa response in humans with essential hypertension and in hypertensive cats points to the possibility of a similar underlying

mechanism. The marked blood pressure rise in the human hypertensives may have resulted from defective renal deaminization due to inadequate arterial blood supply of the kidney parenchyma. Findings indicative of relative renal ischemia in human essential hypertension have been obtained by Goldring, Chasis, Ranges and Smith.[4] Similar evidence of diminished effective blood flow per unit of functioning tubular mass has also been demonstrated by Corcoran and Page,[5] for some but not all patients with essential and malignant hypertension.

From the experiments performed it appears that the pressor response is elicited only after a certain threshold value for l-dopa, and therefore of pressor amine, is reached. Blood pressure elevations were not observed with injections of less than 120 mg of dopa. However, it may well be that the maximum extent of the response during a given period of time may be limited, *i.e.*, after the maximum response has been obtained a greater rise need not necessarily occur with increasing amounts of l-dopa. This is illustrated in the experimental animal. A second injection of l-dopa caused no further augmentation of blood pressure when given during the maximum rise produced by the first injection. This may have been due to an inability of the decarboxylase to produce additional pressor amine during the given period of time, or if more hydroxytyramine were formed, to the fact that a plateau in the blood pressure response had already been reached.[6] The failure of small amounts of l-dopa to cause a blood pressure rise in the 2 patients with marked hypertension may be explained on the same basis. However, the amount of l-dopa given may have been too little to elicit a pressor response. It was considered inadvisable to expose these patients to the possible risk of larger amounts of l-dopa.

Summary. Peripheral intravenous injection of l-dopa was found to produce a marked rise in blood pressure in cats with experimental hypertension; no rise was produced in cats with acute renal ischemia or in normal cats. Intravenous injection of l-dopa in humans with essential hypertension produced a similar pressor response; a much less marked pressor effect was obtained in humans with normal blood pressure. The possibility is discussed of defective renal deaminization due to kidney ischemia as being responsible for the marked pressor response to l-dopa injections in essential human hypertension.

The authors wish to thank Dr. Harry Sobotka for valuable guidance and helpful criticism.

[4] Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W., *J. Am. Invest.*, 1941, **20**, 637.

[5] Corcoran, A. C., and Page, I. H., *Proc. Am. Physiol. Soc.*, 1942, **1**, 17.

[6] Ansari, M. Y., *Quart. J. Exp. Physiol.*, 1942, **31**, 161.

13832

Effect of Exercise on Rats Fed a Diet Deficient in Potassium.

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The production of myocardial necroses and changes in the renal tubular epithelium have been reported in several species when animals are placed on a diet deficient in potassium (K).[1,2,3,4] In our experience[4] lesions first appear in the heart and kidneys of rats after the animals have been on the K-low diet for 8 days. No changes have been encountered in the voluntary muscles of any of the rats we have studied. In K-deficient mice calcification of striated muscle fibers has been described when K is added to the diet.[3] The present experiments were designed to determine whether or not severe exercise might produce more extensive cardiac lesions and to find out whether or not changes could be made to appear in the skeletal musculature by this means. Since Heppel[5] has shown that there is a loss of K when muscle is electrically stimulated, it was thought that the store of this cation might be depleted in the muscle fibers and lesions, similar to those found in the heart, might possibly be produced.

The diet used has been described in detail elsewhere.[6] * Fifteen rats 4 to 5 weeks of age were divided into 3 groups, 2 of which were placed on the K low regimen while the third group was used as controls, K being added to the diet. After a preliminary period of 2 weeks on the respective diets, one of the deficient groups and all the controls were made to exercise 6 days a week. They were placed in a large tank of water kept at approximately body temperature. Ten g weights were fastened to their trunks and they then were forced to swim vigorously for an hour the first week and from 90 to 120 min during the 3 ensuing weeks. They were removed from the tank when exhausted. The animals were sacrificed after being on the diet for 6 weeks. Autopsies were performed and 3-4 blocks of the heart and voluntary muscles were studied in each animal. The kidneys also were studied histologically.

The growth of the rats was similar to that which has already been described when such animals are placed on this diet.[6] No significant differences in growth were noted between the exercised and non-exercised deficient animals. The former gained on an average 30 g during the 6 weeks, while the latter increased 32 g in weight; the controls gained 55 g.

Following exercise during the latter half of the experiment the behavior of the deficient animals was interesting. They seemed more exhausted than the controls. When removed from the water, however, they did not stay relatively still in the cage as did the controls, but exhibited spontaneous tetanic contractures of the muscles of the extremities. That is, when they were placed on the floor, after being removed from the tank, they jumped into the air to a height of about a foot. These spasmodic contractures were repeated 10 to 12 times after which the animals lay exhausted. The sudden cessation of movement following removal from the tank seemed to be the inciting factor in bringing on these muscular spasms.

When the voluntary muscles of the deficient animals were examined no lesions were found. In the myocardium, however, there

1 Schrader, G. A., Prickett, C. O., and Salmon, W. D., *J. Nutrition*, 1937, **14**, 85.

2 Thomas, R. M., Mylon, E., and Winternitz, M. C., *Yale J. Biol. and Med.*, 1940, **12**, 345.

3 Liebow, A. A., McFarland, W. J., and Tennant, R., *Yale J. Biol. and Med.*, 1941, **13**, 523.

4 Follis, R. H., Jr., Orent-Keiles, E., and McCollum, E. V., *Am. J. Path.*, 1942, **18**, 29.

5 Heppel, L. A., *Am. J. Physiol.*, 1940, **128**, 440.

6 Orent-Keiles, E., and McCollum, E. V., *J. Biol. Chem.*, 1941, **140**, 337.

* The liver concentrate was kindly supplied by Dr. Guy W. Clark of the Lederle Laboratories. Alpha tocopherol was kindly supplied by Dr. D. F. Robertson of Merck and Company.

were characteristic changes which have been described elsewhere.[4] The lesions were definitely more extensive in the exercised than the unexercised group. The controls showed no lesions. Changes of the same degree were found in the kidneys of the 2 deficient groups while no renal changes were found in the controls.

Since the K content of the muscles of K deficient animals is lowered,[6] it had been hoped that exercise, by causing a further reduction of this cation, might lead to anatomical changes in the skeletal muscle fibers. While this study was in progress a report appeared by Miller and Darrow[7] which showed that voluntary exercise similar to that we had employed did not significantly lower the K content of muscle (per unit of fat-free tissue) of rats, even on a diet deficient in this cation. It thus appears that our negative histological findings are in keeping with the chemical studies of the above investigators.[7]

It should also be pointed out that Carnes *et al.*[8] found no changes in the voluntary muscles of rats which had received large amounts of desoxycorticosterone acetate. These authors also pointed out a difference between the response of the rat and the dog to this hormone. In the latter animal a syndrome resembling familial periodic paralysis has been produced following large amounts of adrenal cortical hormone.[9] Studies of the muscles of other such animals on low K diets are thus needed.

The peculiar physiological response that our animals showed following a cessation of exercise is difficult to explain. It consisted of a series of spasmodic, violent contractures that individually did not last abnormally long. Thus it is not myotonia. It does illustrate again the important relationship that K bears to muscular contraction.

The added burden of exercise on the hearts of these deficient animals led to more extensive changes than in the unexercised deficient groups. Chemical studies on the myocardium such as carried out by Miller and Darrow[7] on voluntary muscle would be of interest in this connection to determine whether or not the heart muscle fibers lost K following severe exertion and if their K content could be correlated with the extent of the lesions. A decrease in the concentration of K in the myocardium of rats on a low K diet has been reported by Orent-Keiles and McCollum.[6]

Summary. A study of the effect of excessive voluntary exercise was made in animals maintained on a low K diet. No changes were encountered in the voluntary muscles; the myocardium of the exercised group showed more extensive lesions than that of the controls. Peculiar tetanic contractures of the extremities were observed in the K-deficient animals following severe exercise.

7 Miller, H. C., and Darrow, D. C., *Am. J. Physiol.*, 1941, **132**, 801.

8 Carnes, W. H., Ragan, C., Ferrebee, J. W., and O'Neill, J., *Endocrinol.*, 1941, **29**, 144.

9 Ragan, C., Ferrebee, J. W., Phyfe, P., Atchley, D. W., and Loeb, R. F., *Am. J. Physiol.*, 1940, **131**, 73.

13833 P

Estimation of Trypsin by Fibrinolysis.

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It has been pointed out that the actions of crystalline trypsin on blood-clotting systems are: 1. thromboplastic,[1] 2. prothrombinolytic[2] and thrombinolytic,[3] 3. fibrinogenolytic and fibrinolytic.[4] Preliminary tests with varying amounts of crystalline trypsin (X.T. = preparation of Dr. T. E. Weichselbaum, Washington University, St. Louis) and commercial trypsin (F. T. = Fairchild Bros. and Foster) established the fact that fibrinogen and fibrin are more sensitive than thrombin to the lytic action of the protease. This is a serious handicap in attempts to assay trypsin by the thrombinolytic method previously suggested.[3] However, fibrinolysis offers an alternative and simpler technic for the enzyme assay, which may be made especially accurate if the fibrinolysis is followed by *relative turbidimetry* (*cf.*[4]) using the Evelyn photoelectric colorimeter.

Method. Essential reagents include a stable fibrinogen (F)[4] and a stable thrombin (*e.g.* T_G = 1:100 dilution of "rabbit clotting globulin," kindly supplied by Dr. I. A. Parfentjev,[5] Lederle Labs., N. Y.). In each of a series of enzyme dilutions, 1 cc trypsin is added to 5 cc F + 3 cc T_G + 1 cc saline (= control for experiments, other than those cited, in which it is desired to test effects of inhibitors, etc.), a few sec prior to the onset of clotting. Control of clotting conditions must include temperature and pH. Relative turbidity is measured at intervals by the galvanometer scale deflection of the Evelyn apparatus. Data are plotted in the form of a series of curves corresponding to the various dilutions of trypsin used. It is not the shape of the curves which is significant but their relative positions along the time axis. Hence, conversion of the galvanometer reading (G) into "photometric density" (L), by means of the formula L = 2—log G, is an unnecessary refinement of the method described.

Quantitation of tryptic fibrinolysis, timed with the photoelectric colorimeter. In order to determine the sensitivity of the "lytic-time" differences over a convenient range of observation times, dilutions varying by 10% were made from a stock trypsin solution (1:2000 F.T. = 100%). The data (G values) between 90% and 20% are illustrated in Fig. 1. The sharply rising end portions of the fibrinolytic curves are separated by marked time intervals.

An unknown solution (suitably diluted) of similarly-acting lytic agent could easily be assayed, well within 10%, from the position of its end-curve relative to the trypsin standards. Additional tests with crystalline trypsin indicate an absolute (practical) sensitivity of the test of the order of 1:100,000 X.T. (1:1,000,000 *final* conc.), depending, in part, on fibrinogen concentration, pH, and other factors which remain to be investigated. The method is suitable for serum tryptase, papain, and other proteases (*e.g.* venoms, bacterial fibrinolysins, etc.) which are active in a pH range (6.5 — 8.5) suitable for the thrombic-clotting of fibrinogen. It is an easy way to follow steps in the purification of such enzymes and their behavior with respect to small changes in pH, salt concentration, action of inhibitors, etc.

In the tests of Fig. 1 the following *clotting-times* were noted: (a) control (0) — 30 sec.; (b) other tubes — less than 1-2 min. Thus, neither thrombinolysis nor fibrinogenolysis were complicating factors of any practical

1 Ferguson, J. H., and Erickson, B. Nims., *Am. J. Physiol.*, 1939, **126**, 661.

2 Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 80.

3 Glazko, A. J., and Ferguson, J. H., *J. Gen. Physiol.*, 1940, **24**, 169.

4 Ferguson, J. H., *J. Gen. Physiol.*, 1942, **25**, 607.

5 Parfentjev, I. A., *Am. J. Med. Sci.*, 1941, **202**, 578.

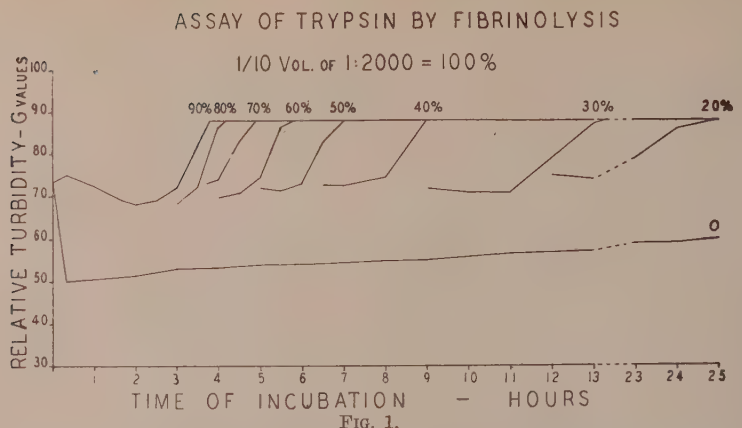

FIG. 1.

Temp. = 29°C; pH = 7.95. G values, with green filter (540): tube and saline (10 cc) = 100; initial reading (all tubes) = 73.0; final reading (all tubes, except control) = 88.0. The persistent turbidity is probably due to the faint opalescence attributable to lipids.

significance. Such fibrinogenolysis as occurs lessens the significance of the absolute turbidimetric values but, within limits, enhances the separation of the test curves. The method is valid, therefore, provided that a good clot is obtained within a minute or two of the control. The stock trypsin solution in the cited series could, with advantage, have been a little less dilute. With a 1-2 hr "lytic-time" for the 100% standard, the series should be completed, down to 20% or 10%, in 6-10 hr.

A good commercial trypsin affords a satisfactory standard of reference but it should be analyzed by some independent standard technic, such as the Anson and Mirsky[6] hemoglobin method. This was not done for the preparation cited, but four "end-points," *viz.* 1. incipient thrombinolysis in 1 hr, 2. complete thrombinolysis in 1-1½ hr, 3. fibrinogenolysis, in 15 sec; 4. fibrinolysis in 2-2½ hr, were obtained in parallel dilution series of (a) F.T. and (b) X.T. The 4 comparisons all placed the strength of F.T. at 0.4 × that of the crystalline trypsin, 1 mg of which assayed 4.24×10^{-3} [T.U.] Hb.

[6] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1933, **17**, 151, *et seq.*

13834

Mechanism of Bradycardia in Rats with Thiamine Deficiency.

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Bradycardia is a well-known phenomenon in thiamine-deficient rats. The following experiment was carried out to determine whether bradycardia could be demonstrated in the isolated auricles of such animals. The effect of addition of thiamine to the isolated preparations was also studied.

Healthy albino rats, adult males, were used. Half were fed the Sherman and Sandels diet,[1] the remainder received the routine laboratory diet. Litter mates were scattered in the two groups.

The thiamine-deficient rats remained on the diet 31 to 40 days. All animals were accustomed to being handled by almost daily weighing. Satisfactory electrocardiograms were obtained by smearing contact jelly on both sides of the animal's body just behind the forelegs; large aluminum electrodes were held in position by the operator who wore rubber gloves. The importance of training in obtaining basal heart rate tracings was evident. Keeping in mind the difficulties encountered by Robertson and Doyle,[2] counts were made at different places in the tracings. The greatest variation in any tracing was 10 beats per min. In most cases the variation was 2 or 3 beats per min. Three to 7 tracings were made on the thiamine-deficient rats and 2 to 6 on the normals.

After the last tracing the animal was killed by a blow on the head, the heart was removed and the auricles were dissected free under iced Ringer's solution. The auricles were then strung up in a Dale bath in oxygenated Ringer's at 35°C. Auricles from a normal and a vitamin-deficient rat were set up in the same bath, and the beats were recorded simultaneously by means of heart levers on a smoked drum.

After allowing the auricles to establish a regular rhythm (15 to 20 min), a control tracing was made. Thiamine chloride in Ringer's was then added. Two concentrations, 1/1,000,000 and 1/100,000 were used, and the preparation was washed twice between each addition. Records were made 5 min after each addition and a final control record was made with Ringer's alone. The pH remained unchanged throughout and was not altered by the additions of thiamine.

A summary of the results is recorded in Table I.

Results. The final mean heart rate of de-

TABLE I.

No. of animals used		Intact animal Heart rate per min		Isolated auricles—beats per min					
		Initial	Last	Control	B_1 1/1,000,000	% Change	B_1 1/100,000	% Change	Control
Normal	13	466	445	234	254	+8	250	+7	233
Deficient	10	505	344	216	236	+9	202	—6	214
Difference			—101 (±31.1)	—18 (±28.5)	—18 (±39.8)		—48 (±27.2)		—19 (±29.6)
T =			3.23	1.23	0.45		1.84		0.74
P =			0.05	0.24	0.65		0.10		0.44

[1] Sherman, H. C., and Sandels, M. R., *J. Nutr.*, 1931, **3**, 395.

[2] Robertson, E. C., and Doyle, M. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 139.

ficient animals was 344 per min, compared to 445 per min for controls. This degree of bradycardia was not reflected, however, in the beat of the isolated auricles, the mean rates for the two groups being 216 per min and 234 per min respectively. Addition of thiamine chloride to isolated auricles caused no greater increase of rate in those from deficient animals than in controls. MacDonald and McHenry[3] have shown that the bradycardia in intact animals is not relieved by administration of thiamine unless food is also given.

The bradycardia would not seem to be a function of metabolic changes in the heart muscle as, for example, the tachycardia of hyperthyroidism, which persists after the auricles are isolated from the intact animal.[4] It is possible that chemical factors responsible for bradycardia might diffuse out of the myocardium during the period of isolation but it is more likely that the bradycardia is of neurogenic origin.

Summary. The bradycardia of thiamine-deficient rats disappears when the auricles are isolated *in vitro*. It is probably of neurogenic origin.

[3] MacDonald, D. G. H., and McHenry, E. W., *Am. J. Physiol.*, 1940, **128**, 608.

[4] Lewis, J. K., and McEachern, D., *Bull. Johns Hopkins Hosp.*, 1931, **48**, 228.

13835

Protection of Mice Against Meningococcal Infection by Sulfadiazine, and Inhibition of Protection by Para-aminobenzoic Acid.

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During the course of an investigation into the immunological response of patients following meningococcal infection,[1] a number of mouse-protection tests were undertaken with serums obtained from patients during the early and convalescent stages of the disease. All of these patients with meningitis or meningococcemia had received treatment with sulfonamides, and most of them received sulfadiazine. It was noted that mice were protected against multiple lethal doses of meningococci by serum containing sulfadiazine, even in amounts less than 1 mg %, regardless of the stage of illness during which the serum was obtained from the patient. The addition of para-aminobenzoic acid to such serum, in a concentration of 5 mg %, had no inhibitory effect on this protection. Similar protective action was exerted by small amounts of sulfadiazine diluted in normal serum or in physiological saline, and again the addition of para-aminobenzoic acid was without effect. Because of these observations, further investigations were carried out in order to determine (1) the amount of sulfadiazine necessary to induce protection of mice against a virulent strain of Group I meningococcus and (2) under what circumstances this protection was inhibited by para-aminobenzoic acid.

Materials and Methods. Three solutions of sodium sulfadiazine in physiological saline were made, containing 1.0, 0.5 and 0.1 mg per 100 ml. Similar concentrations of the drug were prepared in normal human serum which had been diluted 1:5 in saline. These were injected intraperitoneally into 3 groups of mice, in 0.5 ml amounts, so that the doses for each group were 0.005, 0.0025 and 0.0005 mg, respectively. These injections were made 30 min before the injection of organisms.

The following methods were employed for

* Fellow of the Frederick Tilney Memorial Fund.

[1] Thomas, L., Smith, H. W., and Dingle, J. H., submitted for publication.

TABLE I.
Protective Action of Sulfadiazine for Mice Inoculated Intraperitoneally with Group I Meningococci, and Effect of Para-aminobenzoic Acid upon This Protection.

Sulfadiazine mg	Para-aminobenzoic acid	No. of meningococci injected* 100,000	10,000	1,000	100
.005	0	0†	0	0	0
.0025		0	0	0	0
.0005		2	2	2	0
.005	0.1 mg intraperitoneally with sulfadiazine	0	0	0	0
.0025		0	0	0	0
.0005		2	2	1	1
.0025	0.1 mg intraperitoneally with sulfadiazine, followed by 0.5 mg subcutaneously with organisms	0	0	0	0
.005	0.1 mg intraperitoneally with sulfadiazine, and 0.5 mg subcutaneously with organ-	2	2	2	2
.0005	isms, followed by 0.5 mg subcutaneously every 3 hrs for 21 hrs	2	2	2	2
0	0	2	2	2	2

*1.0 ml of a 3% suspension of mucin containing the given number of meningococci was inoculated intraperitoneally 30 minutes after the administration of sulfadiazine.
†Two mice were inoculated in each group. Each numeral represents the number of mice dying.

the administration of para-aminobenzoic acid: (1) 0.1 mg of para-aminobenzoic acid was added to the sulfadiazine before the intraperitoneal injection of the latter. (2) 0.5 mg of para-aminobenzoic acid, dissolved in 0.5 ml of distilled water, was injected subcutaneously into the abdominal wall at the time of the inoculation of organisms, *i.e.*, 30 min after the injection of sulfadiazine. (3) Eight subcutaneous injections of 0.5 mg of para-aminobenzoic acid were made at 3-hr intervals, beginning at the time of the injection of organisms.

Swiss mice, weighing between 15 and 18 g, were used. All were from a single breed. A 3% suspension of hog mucin in distilled water was prepared by mixing in a Waring Blendor, and was then sterilized by autoclaving and adjusted to pH 7.4.

The same strain of Group I meningococcus (strain No. 2) was used in all the tests. This strain was isolated from the spinal fluid of a patient, and had been maintained in the frozen state. Previous titrations of the virulence for mice had shown the 50% lethal dose to be ten organisms. Titration of the *in vitro* action of sulfadiazine against this strain in a synthetic medium[2] had shown that 0.04 mg % of the drug was bacteriostatic, and 0.1 mg % was bactericidal in a culture originally containing 430 organisms per ml.†

Dilutions of the organisms in mucin were made so that the 10^{-7} dilution contained approximately 100 organisms per cm^3. The dilutions employed for the injection of mice were 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. In each experiment, 2 mice were injected intraperitoneally with 1 ml of each dilution of organisms.

The mice were observed for 72 hr after the injection of organisms, although almost all deaths occurred within the first 24 hr. Cultures of the heart's blood were made on all mice shortly after death, and these were positive for meningococci in all instances.

Results. The results were clear-cut and confirmed the previous observations made with the serum from patients. Complete protection against 100,000 organisms, or 10,000 50% lethal doses, was afforded by 0.0025 mg of sulfadiazine, as is shown by the experiment given in Table I. Protection against 100 or-

2 Frantz, I., *J. Bact.*, 1942.

† These experiments were carried out by Mrs. Laura J. Ingraham.

ganisms occurred with 0.0005 mg of sulfadiazine. In 2 other groups of experiments, the same degree of protection by sulfadiazine occurred when the drug was dissolved either in physiological saline or in normal human serum, diluted 1:5. The administration of 0.1 mg of para-aminobenzoic acid with the sulfadiazine did not alter the protection, nor did the additional injection of 0.5 mg 30 min later, at the time of the injection of organisms. When repeated subcutaneous injections of para-aminobenzoic acid were made in 0.5 mg amounts, at 3-hr intervals, however, all of the mice died within 21 hr. Normal control mice injected with the same doses of organisms, but without sulfadiazine or para-aminobenzoic acid, all died within 21 hr. Twelve normal mice, not included in the table, were injected subcutaneously at 3-hr intervals with 8 doses of para-aminobenzoic acid, each of 0.5 mg, but no sulfadiazine was given. All of these mice survived.

Comment. The failure of a single administration of para-aminobenzoic acid to inhibit the protective effect of sulfadiazine may be due to two factors: (1) The rapid excretion of para-aminobenzoic acid from the body in contrast to the relatively slow excretion of sulfadiazine. Experimental evidence of this difference has been reported for man[3,4] and for the rabbit,[5] although comparable studies have not yet been carried out in mice. (2) The extreme sensitivity of the meningococcus to small amounts of sulfadiazine. This is indicated by the *in vitro* tests and also by the fact that mice are fully protected against this particular strain of Group I meningococcus by as little as 0.0025 mg of drug.

The successful inhibition of the action of sulfadiazine by repeated subcutaneous injections of para-aminobenzoic acid may best be explained by assuming that a considerable concentration of the latter drug is maintained for a prolonged period, thus producing a constant inhibitory effect. A primary toxic action of the para-aminobenzoic acid itself is, of course, a factor which must also be considered, although control mice survived parallel injections of para-aminobenzoic acid and the total dose of para-aminobenzoic acid was less than 6.0% of the amount which Scott and Robbins[6] have reported as lethal for mice in a single intravenous injection.

Dr. George F. Badger has kindly tested the statistical significance of the above data and has come to the following conclusion: "Due to the consistent results obtained with the various drug dosages, these results are statistically significant."

The results of these experiments indicate that the mouse protection test is an impractical and unreliable method for assessing the antibody titers of sera from patients who have recently received sulfonamide therapy.

Summary. (1) 0.0025 mg of sulfadiazine is sufficient to protect mice against from 10 to 10,000 50% lethal doses of a virulent strain of Group I meningococcus. (2) The protective action of sulfadiazine is not inhibited by a single administration of para-aminobenzoic acid accompanying the sulfadiazine, nor by a second injection after 30 min. (3) The protective action of sulfadiazine may be inhibited by repeated subcutaneous injections of para-aminobenzoic acid at 3-hr intervals.

This study was aided in part by a grant from the William W. Wellington Memorial Research Fund.

We are indebted to Mrs. Muriel B. Stone and to Miss Marguerite Buckingham for technical assistance.

[3] Strauss, E., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1941, **20**, 189.

[4] Peterson, O. L., Strauss, E., Taylor, F. H. L., and Finland, M., *Am. J. M. Sc.*, 1941, **201**, 357.

[5] Harrow, B., Mazur, A., and Sherwin, C. P., *J. Biol. Chem.*, 1933, **102**, 35.

[6] Scott, C. C., and Robbins, E. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 184.

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Intestinal Motility as Influenced Through Extrinsic Nerves as a Result of Central Stimulation by Bulbocapnine.

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Bulbocapnine has been shown to stimulate both sympathetic and parasympathetic centers[1] and to change the level of blood sugar of rats by way of the vago-insulin and sympathetic-adrenal systems. The present study was undertaken to observe the effect of this drug on intestinal motility and rate in dogs. This rate has been shown to be very constant for any given level of intestine.[2-7]

Methods. Trained dogs with exteriorized loops of small intestine in continuity, with blood supply intact, enclosed in bipedicled tubes of skin[8] were used. Records were made from the loops by a 2 tambour air-displacement system. Group 1 had intact nerve supply, Group 2 bilateral vagotomies, Group 3 bilateral splanchnicotomies. The dogs were fasted 18 hr. Control readings for character and rate of intestinal contractions were obtained at the start of each experiment. Intestinal contractions were recorded continuously. Blood sugar levels (Folin-Wu) were determined as controls and after bulbocapnine at 15, 45, and 90 min, and later as indicated.

Each group of animals contained some whose loops were located just below the duodenum and others with loops placed just above the ileo-colic sphincter. These levels may behave differently.[9] Bulbocapnine phosphate was administered subcutaneously, usually in doses of 25-30 mg/kg.

Results. Group 1. Normal dogs. Six animals were used. In 5 of these there was a complete inhibition of intestinal activity. The remaining dog showed increased amplitude of contractions. There was no correlation between increased amplitude of contractions and fall in blood sugar, or vice versa. For instance, of the dogs showing the 2 highest elevations of blood sugar levels after bulbocapnine, the intestine of one was inhibited and the amplitude of intestinal contractions of the other was augmented. Intestinal effects usually preceded the changes in blood sugar levels. The established rate of contraction was not changed in any instance.

Group 2. Splanchnicotomized dogs. Seven animals made up this series. After bulbocapnine all showed an increase in amplitude of intestinal contractions. Blood sugar levels were unchanged in 6 of 7 cases. In the seventh animal there was an elevation of 26 mg %. Rates of intestinal contraction were uninfluenced.

Group 3. Vagotomized dogs. Five dogs comprised this group. Amplitude of contraction of loops of intestine at low levels was inhibited after bulbocapnine, those located high in the jejunum presented increased amplitude of contractions. Blood sugar was elevated in 4 cases, unchanged in a fifth. There was no change in the rate of contraction.

Two dogs with normally innervated Thiry-Vella fistulæ of the jejunum (recorded by a

1 Feldman, J., Cortell, R., and Gellhorn, E., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 157.

2 Oppenheimer, M. J., and Mann, F. C., *Am. J. Digest. Dis.*, 1941, **8**, 86.

3 Oppenheimer, M. J., and Mann, F. C., *Am. J. Digest. Dis.*, 1941, **8**, 90.

4 Douglass, D. M., and Mann, F. C., *Am. J. Digest. Dis.*, 1939, **6**, 318.

5 Douglass, D. M., and Mann, F. C., *Am. J. Digest. Dis.*, 1939, **6**, 434.

6 Douglass, D. M., and Mann, F. C., *Am. J. Digest. Dis.*, 1940, **7**, 53.

7 Castleton, K. B., *Am. J. Physiol.*, 1934, **107**, 641.

8 Biebl, M., *Klin. Wchsch.*, 1930, **9**, 1674.

9 Oppenheimer, M. J., and Glyer, N. M., *Am. J. Digest. Dis.*, 1941, **8**, 471.

distending balloon) were tested for comparison. Although blood sugar was elevated in both, in neither case was the character or rate of contraction of the intestine changed. A dog with a completely denervated exteriorized loop[8] showed no changes in blood sugar levels nor in intestinal activity after bulbocapnine.

There was a definite threshold for dosage with bulbocapnine. Twenty mg per kg which produce catalepsy, are ineffective for blood sugar and intestinal motility. Twenty-five to 30 mg per kg always produced a change in amplitude of intestinal activity, usually after a short period of hyperkinesia.

Hippus of the pupil and alternating tachycardia-bradycardia were added evidence of mixed sympathetic and parasympathetic activity.

Discussion. In normal rats the blood sugar is uniformly elevated after bulbocapnine.[1] In dogs this level may either rise or fall. Evidently in the latter test animal sympathetic predominance is by no means uniform. The intestine is, in contrast, regularly inhibited. When vagus influences act alone the blood sugar level is usually unchanged while the amplitude of intestinal activity was uniformly augmented. This finding may be compared with that in rats where the blood sugar level is depressed 15 mg % in adrenalectomized cases.[1] Lastly, when the splanchnics act unopposed blood sugar is elevated in most cases. However, intestinal segments highly placed showed augmented amplitude while those situated lower down were inhibited. In rats after bulbocapnine there was a large elevation of blood sugar if the vagi were cut.[1] Hence, as far as the intestine is concerned the splanchnic mechanism gives variable results depending on the level of intestine studied.

Thus it may be pointed out that as a result of central stimulation by bulbocapnine the intestinal responses and blood sugar fluctuations may behave differently, even though both are activated by the same dual (Group 1) or single (Group 2 and 3) efferent mechanism. The vagus effect on motility may be similar to the high intravesical pressures found in the urinary bladder.[10] One can better understand the increased activity of highly placed intestinal loops as a result of sympathetic stimulation when it is pointed out that bulbocapnine causes a peripheral vasodilatation[11] which may reverse the usual action of the extrinsic nerves.[12]

Thiry-Vella fistulæ show no effect after bulbocapnine possibly because distension markedly influences reflex responses.[13] The Biebl loop,[8] as used in this study is recorded without distension.

Threshold observations confirm the work of Spiegel.[14] Bulbocapnine, like several other agents,[2–7, 9] did not change the rate of intestinal contraction.

Conclusions. 1. Although bulbocapnine acts centrally and stimulates both sympathetic and parasympathetic mechanisms, in dogs it showed qualitative and quantitative differences between its effects upon the level of blood sugar and upon intestinal activity. 2. In normal dogs under bulbocapnine either mechanism may predominate in respect to blood sugar levels. Sympathetic effects were most important for intestinal motility. Amplitude of contraction was diminished. 3. In vagotomized dogs loops of intestine placed just below the duodenum showed augmented amplitude of contraction after bulbocapnine, those placed just above the ileo-cecal valve were inhibited. Blood sugar was elevated in most cases. 4. In splanchnicotomized dogs the vagus mechanism is activated by bulbocapnine in respect to amplitude of intestinal contractions but not for blood sugar levels. 5. Bulbocapnine does not change the rate of intestinal contractions.

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[11] Molitor, H., *J. Pharm. Exp. Therap.*, 1938, **62**, 16.

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13837

Action of *Triturus* Toxin on the Heart of the Frog.

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Since Twitty and coworkers[1] demonstrated the existence of a powerful toxin in the eggs and embryos of the amphibian *Triturus*, investigations have been conducted to determine its physical and chemical characteristics and physiological effects.

Horsburgh, Tatum and Hall[2] showed that the toxin produces death in mammals, usually preceded by convulsions, by direct depression of the central nervous system. These workers demonstrated that while paralysis of the respiratory nerves and muscles occurs as a result of depression of the centers, the toxin paralyzes somatic motor nerves and skeletal muscle generally. Vagus and splanchnic nerve responses are abolished, but not, according to the evidence obtained, those of the cervical sympathetic trunk. The experiments gave no evidence of an effect on cardiac or smooth muscle since arterial pressure responses to adrenalin were not affected. The arterial fall in pressure was attributed to vasomotor paralysis.

Fuhrman and Field[3] demonstrated that the toxin has little effect on the oxidative metabolism of nervous tissue in experiments treating rat brain *in vitro* with the toxin.

Although Horsburgh, Tatum and Hall[2] were unable to observe any effects of the toxin in the concentrations used on cardiac tissue, it seemed of interest to determine whether at higher concentrations the toxin might not have some direct effect on the beating heart. It seemed possible that further light might be cast on the action of the toxin by testing its effects on rhythmically contracting mechanisms in which it might be possible to separate effects on the pacemaker, on conduction and on contractility. Accordingly both mechano- and electrocardiograms were made of the heart of *Rana pipiens* under treatment with toxin.

The toxin, kindly provided by Dr. Tatum, was dry and had been computed to be of a potency of approximately 3500 mouse units per g. This unit was arbitrarily chosen and defined by Horsburgh, Tatum and Hall[2] as the amount of toxin that killed a 20 g mouse in 10 min. Test solutions were made by adding the dry toxin to frog Ringer's. Although the weight of the toxin was accurately computed and the approximate potency recorded for each test solution, the meager knowledge of the chemical nature of the toxin necessarily limited this work to a qualitative analysis of its effects on the heart. It was observed that over a space of a few months the effectiveness of the dry toxin, even though it was kept on ice, decreased markedly. It was thus noted that in the earlier tests solutions made up to contain .125 m.u. per cc of perfusate had a marked effect, while in later experiments the concentration had to be doubled to duplicate the former results.

Tests were run on the isolated heart in a perfusion chamber, on hearts *in situ* cannulated in the left auricle and on hearts *in situ* with the postcava cannulated. The latter technique produced the most uniform results and gave more normal electrocardiograms.

The first characteristic reaction to occur under treatment with adequate concentrations of toxin was ventricular stoppage in diastole. Direct electrical stimulation showed that the quiescent ventricle retained its contractility but that on continued treatment the contractility was gradually lost. Continued treatment of the heart in which ventricular stoppage had occurred next brought about

[1] Twitty, V. C., and Elliott, H. A., *J. Exp. Zool.*, 1934, **68**, 247; Twitty, V. C., and Johnson, H. H., *Science*, 1934, **80**, 78; Twitty, V. C., *J. Exp. Zool.*, 1937, **76**, 67.

[2] Horsburgh, D. B., Tatum, E. L., and Hall, V. E., *J. Pharm. and Exp. Therap.*, 1940, **68**, 284.

[3] Fuhrman, F. A., and Field, John, 2d, PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 435.

cessation of sino-auricular activity. This ventricles- auricles-sinus order of stoppage was characteristic. In a number of tests after complete stoppage had been brought about in the heart, washing with Ringer's brought back normal beat, and as was expected, the resumption of sino-auricular activity preceded that of auriculo-ventricular activity.

Electrocardiograms of hearts treated with concentrations inadequate to produce stoppage showed a decrease in rate and a lengthening of the P-R interval. If concentrations great enough to produce stoppage were used, there occurred first a lengthening of the P-R interval and later disappearance of the QRST complex with transient persistence of the P wave. Finally the P wave disappeared. The disappearance of the QRST complex coincided exactly with the observed cessation of ventricular activity. Recovery in Ringer's showed the same reversal of activity as did the mechanocardiograms. It was noted in both mechano- and electrocardiograms that low concentrations brought about a decrease in rate, while with high concentrations no perceptible decrease in rate occurred before cessation of activity.

To conclude, it may be said that *Triturus* embryonic toxin primarily affects the conducting mechanisms in the frog heart and secondarily contractility. Auriculo-ventricular conduction is first impaired, then sino-auricular activity, and lastly the contractility of the heart muscle itself.

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Estimation of Tryptophane Content of Various Proteins.

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An extensive study of the tryptophane content of various proteins was made by Jones, Gersdorff and Moeller.[1] They employed the Holm and Greenbank[2] modification of the May and Rose[3] method, in which the protein, together with a solution of paradimethylaminobenzaldehyde in 17.5% hydrochloric acid is incubated at 37° until maximum intensity of color develops. This usually requires from 6 to 8 days. Comparison is made with a standard casein similarly treated.

The desirability of shortening the time required for maximum color development led Bates[4] and Sullivan, Milone and Everitt[5] to propose modifications based on the suggestions of Komm[6] and of Boyd,[7] involving the use of mild oxidizing agents as accelerators. Bates[4] adds sodium nitrate to the mixture of casein and reagent in hydrochloric acid, and obtains maximum intensity of color very speedily at room temperature. Sullivan, Milone, and Everitt[5] digest the mixture of protein and reagent in 17.5% hydrochloric acid at 85° for 15 min, add a 0.3% solution of hydrogen peroxide, and read after cooling to room temperature.

The short procedure of Sullivan *et al.*[5] would permit the rapid estimation of the tryptophane content of a large number of proteins. Accordingly, 20 proteins, obtained, with the exception of egg albumin, from Jones,[1] were analyzed by this method. This permitted the comparison of the results so obtained with those reported by Jones, Gersdorff and Moeller.[1]

1 Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, 1924, **62**, 183.

2 Holm, G. E., and Greenbank, G. R., *J. Am. Chem. Soc.*, 1923, **45**, 1788.

3 May, C. E., and Rose, E. R., *J. Biol. Chem.*, 1922, **54**, 213.

4 Bates, R. W., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, 1937, **119**, vii.

5 Sullivan, M. X., Milone, H. S., and Everitt, E. L., *J. Biol. Chem.*, 1938, **125**, 471.

6 Komm, E., *Z. physiol. Chem.*, 1926, **156**, 35.

7 Boyd, W. J., *Biochem. J.*, 1929, **23**, 78.

Experimental. The colored complex formed as a result of the reaction of para-dimethylaminobenzaldehyde with casein in the rapid procedure, and that formed by casein and by tryptophane in the long procedure were analyzed, using a Coleman Regional Spectrophotometer, Model 10 S. The results, when plotted, yielded 3 curves exhibiting the same general absorption characteristics, with maximum absorption at a wave length of 590 mμ in each case.

The tryptophane content of 20 proteins was determined by the rapid procedure,[5] using casein as a standard. The casein, obtained from the Will Corporation, was analyzed by the Holm and Greenbank[2] method. The first standardization of this sample was carried out in June 1940 and showed 2.44% tryptophane, after correcting for moisture and ash. Subsequent standardizations over a period of 2 years showed no material variation. Hence there is no need to restandardize a given sample of casein, once its tryptophane value has been determined.

TABLE I.
Percentage of Tryptophane in Various Proteins.

Natural source and name of protein	Found by authors	Reported by Jones *et al.*
Cow's Milk		
Casein	2.44	2.2
Lactalbumin	2.69	2.69
Hen's Egg		
Ovalbumin	1.77	2.25
Hemp Seed		
Edestin	2.56	2.48
Wheat		
Gliadin	1.10	1.09
Soy Bean		
Glycinin	1.78	1.66
Mung Bean		
Alpha-globulin	1.97	2.03
Beta-globulin	1.12	1.18
Navy Bean		
Phaseolin	1.76	0.94
Conphaseolin	2.14	2.79
Lima Bean		
Alpha-globulin	1.96	1.92
Beta-globulin	1.58	2.16
Locust Bark		
Albumin	3.29	4.18
Globulin	3.19	3.17
Peanut		
Arachin	1.80	0.88
Conarachin	1.78	2.13
Chinese Velvet Bean		
Stizolobin	1.22	1.36
Tomato Seed		
Beta-globulin	1.23	1.45
Coconut		
Globulin	1.39	1.25
Almond		
Globulin	1.45	1.37
Flax Seed		
Globulin	3.95	3.98

The results presented in Table I represent, for each protein, an average of at least 4 separate determinations on solutions of the same concentration. All values are corrected for moisture and ash.

Discussion. The short procedure herein employed permits the rapid estimation of the tryptophane content of a large number of proteins. It requires the use of casein or some other protein as a reference standard. Sullivan, Milone and Everitt[5] found that although tryptophane itself could not be used, because of its instability in hot acid, casein standardized against tryptophane by the method of Holm and Greenbank[2] could be used, since equivalent amounts of casein tested by both the long and short procedures yielded the same maximum intensity of color. The color developed by casein in the rapid procedure and by both casein and tryptophane in the long procedure, when analyzed spectrophotometrically exhibited the same general absorption characteristics with maximum absorption in each case at 590 mμ.

The results obtained by the short procedure are in fair agreement with those found by Jones, Gersdorff and Moeller.[1] The lower value for egg albumin may be due to the fact that our sample was a commercial preparation of unknown purity. Of the remaining 19 proteins, 13 agree within 10% of the values of Jones *et al.*,[1] obtained by the long procedure of Holm and Greenbank.[2] The remaining 6 proteins for the most part gave lower values than found by these workers, a phenomenon we are at present unable to explain.

Summary. 1. The colored complexes formed as a result of the reaction of pure tryptophane[2] and of casein[2,5] with para-dimethylaminobenzaldehyde appear to be similar. 2. The rapid procedure[5] when employed in the determination of the tryptophane content of various proteins gives results which are in fair agreement with those reported by other investigators[1] using a long procedure.[2]

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A Method for the Reclamation of Agar.

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The possibility that a shortage of agar may occur in the near future has prompted experiments to devise a simple method for the reclamation of used agar media. Methods of reclamation reported by several German bacteriologists during World War I have not proved entirely satisfactory in the Laboratory, Station Hospital, Fort Bragg, North Carolina.

It has been found that plain nutrient agar media, which are extensively used, may be easily and satisfactorily reclaimed by employing an alternate freezing and thawing technic. Probably this process is not feasible from a commercial standpoint. However, it has proved successful where one is concerned with relatively small quantities. With simple modifications the procedure can be adapted to the purification of Endo's agar, eosin-methylene-blue agar, or any other dye containing agar media. Although not yet attempted, agar media containing sugar can probably be similarly reclaimed. Blood agar medium may be treated in the same manner as plain nutrient agar.

The procedure for reclamation of plain nutrient agar without added dye is here described. Pool the agar medium to be processed and autoclave at 15 lb. pressure for 15 min. Adjust the reaction to pH 7.0. Place the melted agar in an ice cube tray and permit to solidify at room temperature. Make parallel cuts lengthwise and crosswise of the agar mass. Freeze completely in the freezing compartment of an electric refrigerator. Break the frozen mass into conveniently sized pieces, place in a gauze sack and let thaw at room temperature. The water carries with it much of the unused soluble ingredients and impurities leaving partially purified solid agar which is insoluble in cold water. Add sufficient distilled water to double the original volume of medium. Melt thoroughly in a boiling water bath or Arnold steamer and cool to 55°C. Add egg white, with thorough stirring, in the proportion of the white of one egg for each 2 liters of media. Boil until coagulation is complete, then filter through a hot wet absorbent cotton filter. (If available, a filter with steam or boiling water jacket will simplify filtration by permitting use of filter paper). Solidify by standing at room temperature, cut into cubes and freeze in the ice compartment as before. Break up the mass and let thaw in a coarse paper filter. Add sufficient distilled water to the residuum to make one-half the original volume. Then again melt, solidify, cut into cubes, freeze and thaw on coarse paper filter as before. Air dry the resultant agar masses at 55°C.

For the reclamation of agar media containing dye, it is advisable, after the first freezing and thawing, to place the solid agar in a washed salt or sugar sack and dialyze in running water for 12 to 24 hr. Most of the dye will be removed by this treatment. If the agar is to be used again in the preparation of the same dye medium, it is necessary only to complete the process as outlined above. Complete decolorization of the agar may be accomplished, if desired, by boiling with animal charcoal.

Dried agar reclaimed by this procedure was used in the preparation of nutrient agar and compared with standard nutrient agar (Difco). Preliminary experiments have shown no significant difference in these two media when used in parallel plate count determinations.

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Effect of Sulfur-Containing Compounds on Growth and Hydrogen Sulfide Production by *Bacterium tularense*.*

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The discovery of the growth-promoting property of cysteine and cystine for *Bacterium tularense* by Francis[1] was of great importance, and made the cultivation of this organism simple and practical. The manner in which cystine exerts its action is not, however, even today clearly understood. Francis found the amino acids tryptophane, tyrosine, histidine dihydrochloride, phenylalanine, leucine, lysine dihydrochloride, and glutamic acid hydrochloride ineffective, as were inorganic sulfur compounds such as sodium sulfite, sodium bisulfite, sodium thiosulfate, ammonium sulfate, magnesium sulfate, and also sublimed and precipitated sulfur. Later work has shown that glutathione[2] and sodium thioglycollate[3] when added to appropriate culture media are capable of supporting growth of *Bact. tularense*, so that attention became focussed on the free or potential —SH group in these substances as the possible active radical. Brigham and Rettger[4] observed that hydrogen sulfide was produced by *Bact. tularense* when growing on slants of blood glucose cystine beef infusion agar. The present

TABLE I.
Compounds Studied for Effect on Growth and Hydrogen Sulfide Production by *Bacterium tularense*.

Substance	Source of reference to method of synthesis	Amounts used per 100 cc of medium, mg	Effect: Growth	Effect: H_2S
Thioglycollic acid	Eastman Kodak Co.	66	+	+
l-Cysteine hydrochloride	Eastman Kodak Co.	168	+++	+++
S-Benzyl-*l*-cysteine	Synthetic[5]	174	0	0
N-Benzoyl-*l*-cysteine	Synthetic[6]	184	0	0
Cysteic acid	Synthetic[7]	138	0	0
l-Cystine	Eastman Kodak Co.	100	+++	+++
N,N-Dibenzoyl-*l*-cystine	Synthetic[6]	188	0	0
dl-Homocystine	S.M.A. Corporation	112	0	0
dl-Methionine	S.M.A. Corporation	102	0	0
S-Benzyl-*dl*-homocysteine	Synthetic[8]	184	0	0
S-Benzyl-glutathione	Synthetic[9]	326	0	0
Dibenzyl bisulfide	Eastman Kodak Co.	104	0	0

+ = slight. +++ = moderate to fairly good. ++++ = Maximal observed on blood glucose cystine agar.

* The studies referred to herein were conducted in part under the auspices of the International Health Division of the Rockefeller Foundation by the Department of Preventive Medicine and Public Health and the Nutrition Unit of the Department of Biochemistry and the Department of Medicine of Vanderbilt University School of Medicine, Nashville, Tenn.

1 Francis, Edward, *Public Health Rep.*, 1923, **38**, 1396.

2 Downs, C. M., personal communication.

3 Ransmeier, J. C., and Schaub, I. G., *Arch. Int. Med.*, 1941, **68**, 747.

4 Brigham, G. D., and Rettger, L. F., *J. Infect. Dis.*, 1935, **56**, 225.

5 Wood, J. L., and du Vigneaud, V., *J. Biol. Chem.*, 1939, **130**, 109.

6 Brenzinger, K., *Z. Physiol. Chem.*, 1892, **16**, 552.

7 Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.*, 1927, **72**, 433.

8 du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.*, 1935, **109**, 97.

9 Stekol, J. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 108.

studies were undertaken to investigate the possible correlation between growth and the production of hydrogen sulfide by this organism in the presence of various sulfur-containing compounds.

Methods. Analytically pure compounds used in this study and the concentrations employed are shown in Table I. In these amounts the substances were equivalent in sulfur content to 0.1% cystine. The compounds were dissolved in M/10 phosphate buffer pH 7.2 with calculated amounts of dilute NaOH required to convert them to sodium salts, 1 g of glucose added, and the volume made up to 100 cc with molten beef infusion agar. The mixtures were autoclaved for 12½ min at 15 lb. pressure and 120°C. After cooling to 60°C, 5% of sterile rabbit serum was added, the pH was adjusted colorimetrically to 7.1-7.2, and the media were tubed and slanted. The slants were incubated for about 3 days until the water of syneresis had almost all evaporated, and examined for sterility. They were then inoculated heavily with a loopful of growth from a 24-hr blood glucose cystine agar culture of *Bact. tularense.* Strips of lead acetate paper sterilized by heating 10 min at 160°C were inserted in the tops of the tubes, and the slants were incubated at 37°C for one week. When growth appeared, attempts were made to transfer it on similar media. If there was no definite growth at the end of one week a bit of the heavy inoculum remaining on the original slant was transferred to a fresh slant of the same composition to see whether any adaptation to the new medium was developed. When traces of growth appeared only on the first slant these were considered possibly due to substances carried over with the inoculum. Control slants of all media were streaked with a sterile loop and incubated with lead acetate paper, but no spontaneous evolution of hydrogen sulfide was ever observed.

Five strains of *Bact. tularense* were used. "B-38" was obtained through the kindness of Dr. Edward Francis of the National Institute of Health, who isolated it from a patient in 1920 and maintained it by transfer on blood glucose cystine agar. It is now considered non-virulent. "V-13" was recovered by Dr. G. John Buddingh from the spleen of a patient dying of tularemia at Vanderbilt University Hospital in February 1940. It was virulent when the first studies were made, but during the course of our work it lost the ability to kill mice and chick embryos as described elsewhere.[10] The "Conn" and "Pack" strains were likewise obtained from Francis, and the former was found highly virulent for mice and chick embryos, as was the "Bishop" strain, which was isolated by one of us in culture from pleural fluid of a non-fatal case of tularemia at Vanderbilt University Hospital, March 18, 1942. The "Pack" strain has not been subjected to recent virulence tests.

Results. Using the "B-38" and "V-13" strains, no growth was obtained on media containing amounts of cysteic acid, N,N-dibenzoyl-cystine, methionine, S-benzyl-homocysteine, S-benzyl-glutathione, and dibenzyl disulfide equivalent in sulfur content to 0.1% cystine. With S-benzyl-cysteine, N-benzol-cysteine, and homocystine there was very questionable slight growth about which it was difficult to be sure owing to the heaviness of inoculum used. Only with slants containing S-benzyl-cysteine was very faint darkening of the lead acetate paper observed, which may have been due either to hydrogen sulfide or benzyl mercaptan. Growth could not be transferred on these media. Moderate to fairly good growth was observed on slants containing cystine which, however, never equalled in luxuriance that yielded by the best blood glucose cystine agar, but was maintained without diminution through 4 transfers. Cysteine hydrochloride permitted moderate and thioglycollic acid poor growth, which could nevertheless be transferred on the same media.† Hydrogen sulfide was always formed from cystine, cysteine hydrochloride, and thioglycollic acid in quantities roughly parallel to the amount of growth.

[10] Ransmeier, J. C., unpublished data.

† In a limited number of experiments poor growth and slight hydrogen sulfide production were obtained on media with 64 mg % of sodium sulfide.

Additional media were prepared containing double quantities of cystine (0.2%), cysteine hydrochloride, and thioglycollic acid dissolved in double the former amount of phosphate buffer. In such concentration some of the cystine crystallized out from the finished medium. Growth occurred on these slants but it was somewhat inferior to that noted above. The hydrogen sulfide production again paralleled the growth.

Since Sahyun *et al.*[11] observed optimal growth of *E. coli* in synthetic medium containing cysteine in concentrations of 50 mg % and noted inhibition with 100 mg % or more, it was decided to test lower concentrations of some of the substances studied. Accordingly media were prepared containing half the amounts of methionine, homocystine, and S-benzyl-homocysteine shown in the table. Media with equivalent amounts of cystine (0.05%) and cysteine hydrochloride were heated for 1 hr in an Arnold sterilizer under nitrogen atmosphere to avoid possible oxidation or partial decomposition of these compounds during autoclaving. Using 5 strains of *Bact. tularense,* by far the best growth and hydrogen sulfide production were observed on the cystine and cysteine slants, "V-13" showing somewhat less vigorous performance than the others. Questionable to slight growth appeared with methionine, homocystine, and S-benzyl-homocysteine, but it could not be transferred after 1 week's incubation. No significant amounts of hydrogen sulfide were formed by any of the strains on media containing these three substances.

Summary and Conclusions. 1. Of all the substances studied, cystine, cysteine, and to a much lesser extent thioglycollic acid were the only compounds effective in promoting growth of *Bact. tularense.* 2. Under the conditions described it is confirmed that cystine or cysteine is an essential component of media for cultivation of *Bact. tularense,* as was found previously.[1,4] The effectiveness of glutathione could be ascribed to the cysteine moiety of the tripeptide, while that of thioglycollic acid may not be due to the utilization of the acid *per se* but to some intermediary metabolic product common to cystine and cysteine. 3. Inasmuch as practically no stimulation of growth of *Bact. tularense* by methionine and homocystine was observed, it appears justifiable to conclude that cystine and cysteine were not elaborated from these compounds. It is of interest to note that this is in contrast to results reported with animals.[12-18]

4. Whenever definite growth of *Bact. tularense* was observed hydrogen sulfide was formed. It is not possible at present to state the paths of the reactions leading to hydrogen sulfide production from cystine and cysteine by this organism. It may be that the same mechanism prevails as was postulated by Fromageot[19] and Desnuelle[20] for other bacteria and Smythe[21] for liver tissue. 5. Benzylation of the —SH radical of cysteine and glutathione or benzoylation of the alpha amino groups of cysteine or cystine prevents the utilization of these amino acids for purposes both of growth and hydrogen sulfide production by *Bact. tularense.* These results parallel those obtained in metabolic experiments with animals.[22] Apparently this bac-

11 Sahyun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Infect. Dis.,* 1936, **58**, 28.

12 Jackson, R. W., and Block, R. J., *J. Biol. Chem.,* 1932, **98**, 465.

13 White, A., and Lewis, H. B., *J. Biol. Chem.,* 1932, **98**, 607.

14 Stekol, J. A., and Schmidt, C. L. A., *Univ. Cal. Pub. Physiol.,* 1933, **8**, 31.

15 Virtue, R. W., and Lewis, H. B., *J. Biol. Chem.,* 1934, **104**, 59.

16 Womack, M., Kemmerer, K. S., and Rose, W. C., *J. Biol. Chem.,* 1937, **121**, 403.

17 du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.,* 1933, **101**, 719.

18 du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.,* 1939, **131**, 57.

19 Fromageot, C., and Moubasher, R., *Enzymologia,* 1937, **2**, 121.

20 Desnuelle, P., *Enzymologia,* 1939, **6**, 80, 242, 387.

21 Smythe, C. V., *J. Biol. Chem.,* 1942, **142**, 387.

22 Magnus-Levy, A., *Biochem. Z.,* 1907, **6**, 541; Shiple, G. J., Rose, A. R., and Sherwin, C. P., PROC. SOC. EXP. BIOL. AND MED., 1922, **20**, 360; Lewis, H. B., Updegraff, H., and McGinty, D. A., *J. Biol. Chem.,* 1924, **59**, 59; Jones, J. H., Andrews, K. C., and Andrews, J. C., *J. Biol. Chem.,* 1935, **109**, xlvii.

terium lacks the mechanism for effective splitting of benzyl and benzoyl radicals from the substituted amino acids. 6. Since homocystine did not permit growth or production of hydrogen sulfide by *Bact. tularense* while cystine was highly effective in both these respects, it is apparent that the length of the carbon chain as well as the availability of amino and —SH or S-S groups is of importance in the reactions involved. 7. It is likely that the enzymic potentialities for metabolism by *Bact. tularense* are relatively quite limited. Its tendency to intracellular growth and its biological position of almost if not entirely obligate parasitism suggest that it has come to depend upon its hosts to supply it with certain essential and possibly quite specific utilizable substances, among them cysteine or derivatives thereof. This is in contrast to many other pathogenic bacteria which, while able to attack cysteine with production of hydrogen sulfide, can grow equally well in the absence of this substance. Investigations with a number of such organisms are reported in the next paper.

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Production of Hydrogen Sulfide from Sulfur-Containing Compounds by Various Bacteria. I. Experiments with Beef Infusion Agar Basic Medium.*

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Earlier work has shown that many widely differing microörganisms can produce hydrogen sulfide from cystine or cysteine.[1,2,3] On the other hand, taurine,[1] methionine, and various sulfhydryl, amino, and carboxyl substitution products of cysteine proved to be unavailable for this process.[3] In a previous communication we reported that cystine, cysteine, or to a lesser extent thioglycollic acid appeared to be essential for growth and hydrogen sulfide production by *Bacterium tularense in vitro*. Several substitution products of cystine and cysteine, as well as methionine, were found ineffective in this respect.[4] The present report deals with a similar study extended to other bacteria, some of which have not been thus investigated previously.

Methods. The compounds studied and the sources thereof were the same as those described in the previous paper,[4] and the concentrations used are shown in Table I. The compounds were dissolved in m/10 phosphate buffer pH 7.2 containing dilute NaOH as before, the volume made up to 100 cc with molten beef infusion agar, and autoclaved 12½ min at 15 lb. pressure and 120°C. After cooling to 60°C, the pH was adjusted colorimetrically to 7.1-7.2, and slants prepared. Strips of sterile lead acetate paper inserted in the tops of the tubes were used to indicate hydrogen sulfide production. The bacteria studied are listed in Table I. All cultures were incubated at 37°C for 1 week.

Some of the strains used were from stock collections of Vanderbilt University, many

* The studies referred to herein were conducted in part under the auspices of the International Health Division of the Rockefeller Foundation by the Department of Preventive Medicine and Public Health and the Nutrition Unit of the Department of Biochemistry and the Department of Medicine of Vanderbilt University School of Medicine, Nashville, Tenn.

1 Bürger, Max, *Arch. f. Hyg.*, 1914, **82**, 201.

2 Tarr, H. L., *Biochem. J.*, 1933, **27**, 759.

3 Tarr, H. L., *Biochem. J.*, 1933, **27**, 1869.

4 Ransmeier, J. C., and Stekol, J. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 85.

TABLE I.

H_2S Production by Various Bacteria from Sulfur-containing Compounds Added to Beef Infusion Agar. Results at 24 Hours.

	l-Cysteine hydrochloride	S-Benzyl-*l*-cysteine	N-Benzoyl-*l*-cysteine	Cysteic acid	*l*-Cystine	N,N-Dibenzoyl-*l*-cystine	*dl*-Methionine	S-Benzyl-*dl*-homocysteine	*dl*-Homocystine	Beef infusion agar control
Concentration (mg%)	168	174	184	138	100	186	51	92	56	
Bacteria studied										
*E. coli**	4+	4+ Y‡	tr	0	3+	tr	tr?	tr	tr?	tr
E. coli†	4+	3+ Y-Br	tr?	tr?	4+	0	tr?	tr?	0	tr?
Proteus X-19†	4+	4+ Y-Br	2+	1+	4+	tr	1+	1+	2+	tr
Proteus OX-2†	4+	3+ Y-Br	2+	1+	4+	1+	1+	1+	2+	1+
K. pneumoniæ, type B*	2+	3+ Y	2+	0	2+	0	0	tr	0	0
E. typhosa (Brown)*	4+	3+ Y	0	0	3+	0	0	0	0	0
E. typhosa (58)†	4+	2+ Y	0	0	4+	0	0	0	0	0
E. typhosa (H-901)†	4+	3+ Y	0	0	4+	0	0	0	0	0
E. typhosa (O-901)†	4+	2+ Y	0	0	4+	0	0	0	0	0
*S. paratyphi**	4+	2+ Y	0	0	4+	0	0	0	0	0
S. paratyphi (NIH)†	4+	3+ Y	0	0	4+	0	0	0	0	0
S. paratyphi (Dilliard)†	4+	3+ Y	0	0	4+	0	0	0	0	0
S. schottmuelleri (NIH)†	4+	3+ Y-B	0	0	4+	0	0	tr	tr	0
S. enteritidis†	4+	2+ Y	0	0	4+	0	Not studied			0
S. ambigua (Quarles)*	3+	4+ Y-Br	0	0	2+	0	0	0	0	0
S. newcastle†	3+	1+ Y	0	0	3+	0	0	0	0	0
B. abortus (456)*	1+	2+ Br	1+	tr	1+	1+	1+	1+	tr	1+
*M. tuberculosis**	4+	tr Br	tr?	0	4+	0	Not studied			0
*B. subtilis**	4+	3+ Y-B	tr	0	4+	tr	0	tr	tr	0
*S. aureus**	1+	0	1+	0	1+	0	0	0	0	0
*S. aureus**	1+	tr Br	2+	0	1+	0	Not studied			0
*S. lutea**	2+	1+ Br	0	0	2+	0	0	0	0	0
Uninoculated controls	0	0	0	0	0	0	0	0	0	0

* = Vanderbilt University, † = Tenn. State Health Dept. Lab. Stock strains, NIH = originally from National Institute of Health, ‡Y = yellow, Br = brown, B = black color on lead acetate paper.

having been isolated from patients in the hospital. Others were obtained through the kindness of the Tennessee State Health Department Laboratory. The example of *Mycobacterium tuberculosis* studied was an old laboratory strain of human origin received from Dr. Roy C. Avery of the Department of Pathology. Five per cent glycerin was added to the media for its cultivation, resulting in rapid and abundant growth. Control slants of all media were streaked with a sterile loop and incubated with lead acetate paper, but spontaneous evolution of hydrogen sulfide was never observed, even after liquification and acidification with strong HCl.

Results. Excellent growth occurred on all slants. The results observed after 24 hr incubation are shown in Table I.† It will be noted that moderate to large amounts of hydrogen sulfide were produced from cysteine and cystine by all organisms with the exception of *Brucella abortus* and the two strains of *Staphylococcus aureus*. Maximal hydrogen sulfide production occurred within the first 24 hr in most cases, although with *Klebsiella*

† The readings indicated for *M. tuberculosis* were made on the 5th day, after maximum growth had been obtained.

pneumoniae further increase was noted at 48 hr, and with *Shigella ambigua* and *Shigella newcastle* maximal amounts were recorded after 1 week. In fact, one of the most interesting things about this reaction is the great rapidity with which it is initiated. Definite evidence of the evolution of hydrogen sulfide was noted in several instances,‡ for example, at room temperature 1 to 2 hr after inoculation of the slants. Similar observations with *Proteus vulgaris* were reported by Tarr.[2]

Only slight further increase in the amount of hydrogen sulfide produced from cysteine and cystine by *B. abortus* was noted up to 1 week, and with *S. aureus* no additional change occurred after 24 hr. These organisms thus appeared to differ from the rest in producing relatively small quantities of hydrogen sulfide under such conditions, while *Sarcina lutea* was only slightly more active. Nevertheless it is important to note that hydrogen sulfide was formed from cysteine and cystine slants by all bacteria tested.

A different type of reaction was noted on slants containing S-benzyl-cysteine. During the first 24 hr most of the organisms tested formed in the presence of this compound considerable amounts of a volatile substance which turned the lead acetate paper a bright yellow color. On further incubation the extent of this discoloration usually increased, with part of the paper becoming brown or black. Simultaneously the agar slants with S-benzyl-cysteine became milky and opaque in degree roughly paralleling the amount of discoloration observed on the lead acetate paper. Uninoculated control slants of the same medium invariably remained clear. At the end of 1 week this reaction was quite marked or maximal in the case of all bacteria studied except *M. tuberculosis* and 2 strains of *S. aureus*. These did not produce a definite yellow color, but caused a slight brownish discoloration of the lead acetate paper, while the slants on which they were growing remained clear and translucent. Pending identification, it would be premature to indicate the nature of the volatile product liberated from S-benzyl-cysteine, although *a priori* it seems likely that it may be benzyl mercaptan.

Few organisms produced appreciable amounts of hydrogen sulfide from slants containing N-benzoyl-cysteine. Only in the case of the 2 *Proteus* strains, the 2 strains of *S. aureus*, and *K. pneumoniae* was this definitely in excess of that observed with the beef infusion agar control. With *S. aureus* and *Proteus* the reaction did not progress beyond the point indicated at 24 hr, but with *K. pneumoniae* the amount of hydrogen sulfide produced increased somewhat up to 48 hr and was equal to that formed by this organism from slants containing cysteine or cystine. On the other hand, none of the bacteria tested liberated significant amounts of hydrogen sulfide from N,N-dibenzoyl-cystine slants.

Cysteic acid, methionine, and S-benzyl-homocysteine failed to give rise to hydrogen sulfide or mercaptan with any of the organisms studied.

The two *Proteus* strains were the only organisms which produced small but definite quantities of hydrogen sulfide from homocystine slants. Although the amounts formed appeared greater than with controls, the ability of these organisms to liberate hydrogen sulfide from beef infusion agar alone makes interpretation of this observation difficult.

After 48 hr incubation most of the bacteria studied produced minimal to slight darkening of the lead acetate paper from the control slants of beef infusion agar. At the end of 1 week the paper was unchanged only with *S. ambigua, S. newcastle, M. tuberculosis,* 1 strain of *S. aureus,* and *S. lutea.* Similar small amounts of hydrogen sulfide were often formed by many of the bacteria on prolonged incubation from slants containing compounds for which the reaction is recorded as negative or slight at the end of 24 hr. The *Proteus* strains and *B. abortus* were able to liberate a little hydrogen sulfide from all slants, even within the first 24 hr. It seems likely in such cases, except where marked excess is noted

‡ With *Eberthella typhosa* (58), (H-901), and (O-901), *Salmonella paratyphi* (NIH) and (Dilliard), *S. schottmuelleri* (NIH), and *S. enteritidis, Proteus* X-19 and OX-2.

on certain slants, that the hydrogen sulfide was formed from substances present in the beef infusion agar medium rather than from the compounds added. In order to avoid possible interference from this factor, further studies have been undertaken with synthetic media and will be reported in the next communication.

Discussion and Summary. 1. None of the 22 strains of bacteria studied failed to produce hydrogen sulfide from beef infusion agar slants supplemented with cystine and cysteine. The latter may have been partially oxidized to cystine during autoclaving and incubation. From these data we cannot state whether the reduced or oxidized form of cystine is the immediate precursor for hydrogen sulfide formation. 2. The benzylation of the sulfhydryl group of cysteine did not prevent the desulfurization of the molecule by these bacteria, with the possible exception of *M. tuberculosis* and the two strains of *S. aureus.* The volatile decomposition product of S-benzyl-cysteine which reacted with the lead acetate paper was possibly benzyl mercaptan. 3. Hydrogen sulfide was produced from N-benzoyl-cysteine slants only by *Proteus X-19* and *OX-2, K. pneumoniae,* type B, and the two strains of *S. aureus,* while none of the bacteria was able to metabolize N,N-dibenzoyl-cystine with hydrogen sulfide formation. It appears, therefore, that benzoylation of the alpha amino groups prevents the evolution of hydrogen sulfide from cystine. If as proposed by Desnuelle for *Escherichia coli,*[5] the reduction of cystine to cysteine is a necessary step for the production of hydrogen sulfide, it may be that blocking of the amino groups of cystine prevents the conversion of the disulfide to the sulfhydryl form. This would be contrary to the results of experiments with animals by Lewis, Updegraff, and McGinty[6] who administered N,N-dibenzoyl-cystine and recovered N-benzoyl-cysteine from the urine. Some bacteria are apparently able to use the sulfhydryl group of the benzoylated cysteine for hydrogen sulfide production.

4. None of the bacteria studied utilized the sulfur of cysteic acid, methionine, or S-benzyl-homocysteine for hydrogen sulfide or mercaptan formation. It seems that methionine is not converted to cystine or cysteine by these organisms prior to its possible utilization. Similar conclusions were reached in studies with *Bact. tularense.*[4]

5. The failure of homocystine or S-benzyl-homocysteine to yield hydrogen sulfide or mercaptan with any of the organisms excepting possibly the two *Proteus* strains is in contrast to the results obtained with cystine and S-benzyl-cysteine, and suggests that the length of the carbon chain of the sulfur-containing amino acids is one of the determining factors governing the mechanism of desulfurization.

[5] Desnuelle, P., *Enzymologia,* 1939, **6**, 80, 242, 387.

[6] Lewis, H. B., Updegraff, H., and McGinty, D. A., *J. Biol. Chem.,* 1924, **59**, 59.

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Production of Hydrogen Sulfide from Sulfur-Containing Compounds by Various Bacteria. II. Experiments with Synthetic Medium.

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In the first paper of this series[1] the production of hydrogen sulfide by various bacteria from several sulfur-containing compounds added to beef infusion agar was investigated. Because traces of hydrogen sulfide were often formed by many of these organisms from beef infusion agar without supplements, it appeared desirable to extend these studies further, employing synthetic medium of known composition and purity. Such an approach would eliminate the interfering influences of substances present in the beef infusion agar, and permit an unequivocal interpretation of the results obtained.

Methods. The compounds employed were the same as those described previously[1,2] and the concentrations used are shown in Table I. The composition of the basic medium was as follows: asparagine, 3.0 g; glucose, 1 g; K_2HPO_4, 0.5 g; $MgCl_2$, 0.5 g; KCl, 0.2 g, and distilled water, 1000 cc. The pH was adjusted to 7.8, and the solution autoclaved 15 min at 15 lb. pressure and 120°C. The weighed substances were added to the medium which was then heated in an Arnold sterilizer under nitrogen atmosphere for 1 hr. After cooling, 3 cc of sterile phosphate buffer pH 7.8 were added to each 100 cc of supplemented medium. Six cc of the buffer were used in medium containing cysteine hydrochloride. The bacteria employed were 3 of the Vanderbilt University stock strains used in the previous study,[1] namely *Escherichia coli, Klebsiella pneumoniae* type B, *Bacillus subtilis,* and a pigment-forming laboratory strain of *Pseudomonas aeruginosa.* All grew readily in the unbuffered, unsupplemented synthetic medium, and were carried through

TABLE I.
Effect of Sulfur-Containing Compounds on Bacterial H_2S Production in Synthetic Medium. Results at 72 Hours.*

	l-Cysteine hydrochloride	S-Benzyl-*l*-cysteine	N-Benzoyl-*l*-cysteine	Cysteic acid	*l*-Cystine	N,N-Dibenzoyl-*l*-cystine	*dl*-Methionine	S-Benzyl-*dl*-homocysteine	*dl*-Homocystine	Control medium with buffer
Concentration (mg%)	84	87	92	69	50	94	51	92	56	
Bacteria studied										
E. coli	2+	0	0	0	4+	0	0	0	0	0
K. pneumoniæ, type B	4+	0	3+	0	4+	tr	0	0	0	0
B. subtilis	4+	0	0	†	4+	0	0	0	0	0
P. æruginosa	tr	0	0	†	tr	0	0	0	0	0

*tr = trace, 1+ = slight, 2+ = moderate, 3+ = marked, and 4+ = maximal H_2S production.
† = not studied.

* The studies referred to herein were conducted in part under the auspices of the International Health Division of the Rockefeller Foundation by the Department of Preventive Medicine and Public Health and the Nutrition Unit of the Department of Biochemistry and the Department of Medicine of Vanderbilt University School of Medicine, Nashville, Tenn.

1 Stekol, J. A., and Ransmeier, J. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 88.

2 Ransmeier, J. C., and Stekol, J. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 85.

from 12 to 35 serial transfers prior to inoculation of the test media. The inoculum consisted of 0.1 to 0.2 cc of 24 hr culture introduced into 5 cc volumes of test media distributed in 50 cc Erlenmeyer flasks so as to expose a maximum surface to the air. Sterile strips of lead acetate paper were inserted in the tops of the flasks, the latter incubated at 37°C and examined daily for a period of 5 days. In all cases similar flasks uninoculated failed to show evolution of hydrogen sulfide except with cysteine hydrochloride. Slight but perceptible darkening of the lead acetate paper was consistently noticed with the cysteine controls, suggesting partial decomposition of cysteine under these conditions. All of the uninoculated test media remained sterile. Hydrogen sulfide was never produced by any of the organisms from the buffered unsupplemented basic synthetic medium.

Results. Media containing cysteine or cystine yielded moderate to maximal amounts of hydrogen sulfide with *E. coli, K. pneumoniae,* and *B. subtilis,* as shown in Table I. *P. aeruginosa,* gave rise to traces of the sulfide from these substances. Evolution of hydrogen sulfide was noted at 24 hr, reaching the maximal observed reading often by 48 and always by 72 hr.

Only *K. pneumoniae* produced hydrogen sulfide from N-benzoyl-cysteine, and faint traces from N,N-dibenzoyl-cystine. None of the other bacteria studied were able to liberate sulfide under these conditions.

None of the organisms formed hydrogen sulfide or mercaptan from S-benzyl-cysteine, in contrast to the results obtained when this substance was added to beef infusion agar.[1] It appears that the conditions prevailing in the beef infusion agar in some way permit desulfurization of S-benzyl-cysteine with mercaptan formation. Whether this is due to the presence in the beef infusion agar of a specific catalyst lacking in the synthetic medium cannot definitely be asserted at the present time. The possible role of the vitamins in this reaction invites investigation.

None of the bacteria tested produced hydrogen sulfide from cysteic acid, methionine, S-benzyl-homocysteine, or homocystine. These results are similar to those obtained previously with beef infusion agar.[1]

Moderate to excellent growth was observed with all the supplemented media. It appears that methionine, homocystine, and N,N-dibenzoyl-cystine stimulated growth somewhat, but lack of quantitative criteria prevents definite conclusions in this respect. It is evident that these organisms grow abundantly in synthetic medium with or without the sulfur-containing amino acids or their derivatives, and that the reactions leading to hydrogen sulfide production are not necessarily an expression of the rapidity or extent of growth. This is in contrast to the results obtained with *Bacterium tularense* where cystine, cysteine, or to a much lesser extent thioglycollic acid were found essential for growth *in vitro,* and hydrogen sulfide production always paralleled the extent of growth.[2]

A few observations on pigment formation by *P. aeruginosa* may be noted. Little or no color was produced in buffered or unbuffered unsupplemented synthetic medium, or in the presence of S-benzyl-homocysteine or choline (51 mg %). A moderate muddy brown coloration was observed in medium containing N-benzoyl-cysteine, and none or very pale greenish-yellow tint resulted with N,N - dibenzoyl - cystine. S-benzyl-cysteine yielded moderate yellow-green color. Deep bluish-green pigment was produced with cysteine, cystine, methionine, and homocystine. Apparently benzylation of homocysteine and not of cysteine prevented pigment formation. Further study is needed to elucidate the role of the sulfur-containing amino acids and their substitution products in the mechanism of pigment production by *P. aeruginosa.*

Another observation of interest is that after 34 transfers over a period of 2 months in the basic synthetic medium, *K. pneumoniae* continued to produce prominent capsules, thus confirming the findings of Hoogerheide.[3] It also retained high virulence, killing all of 27 white mice injected subcutaneously with 1 cc of 48-hr culture diluted

[3] Hoogerheide, J. C., *J. Bact.*, 1939, **38**, 367.

from 10^{-2} to 10^{-5}, 6 out of 10 receiving the 10^{-6} dilution, and 7 out of 8 receiving the 10^{-7} dilution. The latter dose contained approximately 8 organisms.

Summary. 1. Production of hydrogen sulfide by *E. coli, K. pneumoniae, B. subtilis,* and *P. aeruginosa* in synthetic media containing cysteine or methionine and their derivatives was studied. 2. With the exception of *P. aeruginosa,* all these organisms formed moderate to maximal amounts of hydrogen sulfide from cysteine and cystine. *P. aeruginosa* produced only traces.

3. Only *K. pneumoniae* produced hydrogen sulfide from N-benzoyl-cysteine. This confirms previous observations made with beef infusion agar basic medium.[1] Benzoylation of the alpha amino group of cysteine does not interfere with desulfurization by this organism, although it prevents the reaction in the case of other bacteria studied. This indicates fundamental metabolic differences prevailing in the organisms investigated with respect to this particular reaction. Additional strains of *K. pneumoniae* and other organisms should be tested under similar conditions.

4. None of the organisms formed hydrogen sulfide or mercaptan from S-benzyl-cysteine, in contrast to the results obtained when this substance was added to beef infusion agar. Hydrogen sulfide was not produced by any of the bacteria from cysteic acid, methionine, S-benzyl-homocysteine, or homocystine. 5. A limited number of observations on pigment formation by *P. aeruginosa* in the presence of cysteine, methionine, and their derivatives was made.

6. A strain of *K. pneumoniae* continued to produce large capsules and maintained high virulence for mice after many transfers in a simple synthetic medium.

13843 P

A Rapid Method for the Determination of Races of *Shigella dysenteriae* Flexner.

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Several investigators have already isolated the polysaccharide fraction of *Shigella dysenteriae.* Kurauchi[1] and Meyer and Morgan[2] isolated specific polysaccharide from the Shiga organism; Kurauchi[1] from the Flexner and Sonne. Spassky and Dannenfeldt[3] used the precipitin test for the diagnosis of *Sh. dysenteriae* infection, but failed to note the specificity of the precipitin reaction, basing their failure on the extreme complexity of the antigenic apparatus of the dysenteric bacillus.

We have tried to develop a practical method for the typing of races of *Shigella dysenteriae* Flexner by using type specific antibodies capable of reacting with the polysaccharide. Antiserum made with the classical Oxford strains did not give clear cut results. However, when freshly isolated strains were utilized, specific reaction was obtained.

Freshly isolated cultures of *Shigella dysenteriae* Flexner strains, classified as W, V, and Z, were utilized. Rabbits were immunized by intravenous injections of saline suspensions of the organisms killed by formalin. A 3-week course of graded doses of dysenteric bacilli was then given to each rabbit. Injections were applied on 3 consecutive days, followed by a rest period of 4 days before starting the next 3 doses. Eight days after the last inoculation, the ani-

[1] Kurauchi (Report of K. Ando), *J. Immunol.,* 1929, **17**, 555.

[2] Meyer, K., and Morgan, W. T. J., *J. Exp. Path.,* 1935, **16**, 476.

[3] Spassky, N. M., and Dannenfeldt, L. A., *Bul. Biol. et Méd. Exp.,* U. S. S. R., 1939, **7**, 202.

mals were bled from the heart and the serum separated aseptically.

Cultures of *Shigella dysenteriae* were isolated from suspected feces, using the technic described by Hardy *et al.*[4] The organisms can be obtained directly from the Krumwiede triple sugar agar.

The polysaccharide fraction of the dysenteric bacteria was extracted by using Fuller's formamide method.[5] To perform the test, 0.1 cc of the antiserum was placed in a small precipitin test tube, an equal amount of polysaccharide solution carefully laid over the serum and the test tube placed in the incubator at 37°C. The precipitin ring usually appeared from 10 to 15 min, sometimes earlier. The test was then applied to one hundred different cultures that had been previously typed by Watt[6] and classified as follows: 29 as W, 34 as Z, and 35 as V. These were treated by the above procedure and their races determined by the precipitin test. In all instances, the classification corresponded to the typing as determined by agglutination, except in 2 cultures, in which there was a difference in the results. The reason for this exception is not yet clear but is the object of study.

Summary. The polysaccharide fraction is a determining factor in the type and group antigens of *Shigella dysenteriae* Flexner. The precipitin test performed by using the polysaccharide fraction and the specific antiserum is presented as a method for classifying the varieties of the group of *Shigella dysenteriae* Flexner.

Recently isolated cultures are essentially necessary for the preparation of type specific antiserums and for the preparation of type specific antigen.

[4] Hardy, A. V., Watt, J., and DeCapito, T., *Pub. Health Rep.*, 1942, **57**, 501.

[5] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

[6] Watt, J., personal communication.

13844

A Method for Determining the Concentration of Penicillin in Body Fluids and Exudates*

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During a study on the effect of penicillin in certain human infections, it at once became apparent that a sensitive, rapid, and simple method for determining the concentration of penicillin in body fluids was desirable. Previously, Fleming[1] used a serial dilution method with *Staphylococcus aureus* as the test organism. Florey[2] found that by using the Oxford-plate method it was possible to determine the concentration of penicillin in both sterile and contaminated fluids. This method also utilized the staphylococcus as the test organism after preliminary dilutions of the unknown solution. The disadvantages of such a method have been commented on recently by Hobby.[3] Foster[4] has shown that the inhibition of growth of the staphylococcus may be measured turbidmetrically and is a function of the concentration of penicillin. This method requires large amounts of the unknown solution and is not readily applicable to the study of body fluids and exudates.

As a preliminary step in our own investigations numerous organisms were tested for

* This study was supported by a grant from the Johnson Research Foundation, New Brunswick, N.J.

[1] Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

[2] Florey *et al.*, *Lancet*, 1941, **2**, 177.

[3] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 227.

[4] Foster, J. W., *J. Biol. Chem.*, 1942, **144**, 285.

TABLE I.
In Vitro Test for Concentration of Penicillin.

Sample	Culture	Serial dilutions in broth: 0.2 cc of sample	1:2	1:8	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Standard penicillin	BB	0	0	0	0	0	0	0	0	+	++
	BAP						0	0	0	++	++
Serum	BB	0	0	0	0	+	++	++	++	++	++
	BAP				0	++	++				

Standard penicillin is an 0.85% solution of sodium chloride containing 20 Florey units per cc.
Serum sample obtained from patient 10 minutes after an intravenous dose of 10,000 Florey units.
Concentration in unknown sample is determined by multiplying the dilution factor by 0.0039.
Culture: BB = blood broth. BAP = blood agar plates. 0 = no visible growth or hemolysis. + and ++ = degrees of hemolysis or growth.
Inoculum: 0.5 cc blood broth containing 1100 streptococci.

their susceptibility to the action of penicillin. It was found that, in general, hemolytic streptococci are from 4 to 16 times more sensitive than staphylococci. An especially sensitive strain of hemolytic streptococcus was therefore chosen as the test organism.†

Methods. The unknown samples of penicillin‡ are stored at 5°C until the time of testing. If the samples are known to be contaminated, sterilization is effected by passing them through a Seitz filter.

To the first 2 tubes of a series of small culture tubes, 0.2 cc of the unknown sample is added. The tubes, with the exception of the first one in the series, contain 0.2 cc of veal infusion broth. From the second tube, then, 0.2 cc of the broth-penicillin sample is removed and serial dilutions are made. In addition, if the solution to be tested is known to contain a very small quantity of penicillin, a further tube containing 0.5 cc of the unknown is added to the test.

A control run with each determination is made up from a standard of penicillin which is stored at 5°C in a solution of 0.85% sodium chloride in a concentration of 20 Florey units per cc. This standard is then treated in a manner similar to the unknown samples.

The test organism is a Group A strain of hemolytic streptococcus obtained from the blood stream of a patient with erysipelas. The appropriate dilution of a 12-hr broth culture is made in veal infusion broth containing 1% erythrocytes so that the final number of organisms varies between 1,000 and 10,000 per cc. The inoculum consists of 0.5 cc of this dilution and is added to each tube as well as to the control series containing dilutions of a known amount of penicillin. The final volume in each instance is 0.7 cc. The cultures are then incubated for 18 hr, following which the tubes are examined for hemolysis. A 3 mm loop of the cultures near the endpoint is streaked on blood agar plates as a check of sterility.

Results. Table I illustrates the results obtained when the test was applied to the serum of a patient 10 min after the intravenous injection of 10,000 Florey units of penicillin. As demonstrated here, 0.0039 Florey unit was required to sterilize the culture. By comparison with the control, then, the serum contained 0.25 Florey unit or 1.25 units per cc.

The above method has been used to test unknown samples of penicillin, whole blood, erythrocytes, urine, spinal fluid, exudates from empyema cavities, and joint fluid. The test has proven to be satisfactory in all instances. Preliminary studies demonstrate that the addition of these various materials does not alter the result of the test. The amount of unknown fluid required is from 0.2 to 0.9 cc which makes the method readily adaptable to clinical studies.

The reliability of the test is well demonstrated by the results obtained in the 30 observations made with the penicillin standard on separate days. In 28 instances the cul-

† Hobby[3] has used the streptococcus to determine the concentration of penicillin.

‡ Penicillin for this study was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, N.J.

tures were sterilized by 0.0039 units and in the remaining 2 by 0.0019 units. The test is subject to the error of serial dilution methods in general.

It is important to store all solutions containing penicillin in the ice box until the time of testing since high temperatures result in a loss of activity. In one experiment a solution of penicillin in saline containing 100 Florey units per cc lost all activity within 7 days when placed at 37°C, whereas the same solution stored at ice box temperature retained its antibacterial effect. Urines which contain penicillin have been stored in the ice box for 2 weeks without loss of penicillin potency.

For those fluids known to be contaminated, sterilization may be effected by passage through a Berkefeld or Seitz filter. This procedure is not attended by any appreciable loss of penicillin.

The sensitivity of the test is increased when a small number of organisms and a small total volume of culture media are used. In general, an inoculum of between 100 and 100,000 organisms is advisable. Variations within this range usually do not alter the results of the test. The addition of 1% erythrocytes to the culture media aids in reading the test. The cultures which show no hemolysis are usually sterile.

Conclusions. A method for determining the concentration of penicillin in various body fluids and exudates is described. It is possible by this method to determine 0.0039 Florey unit per 0.2 cc of solution.

Marjorie Jewell and Thelma Maxon gave valuable technical assistance in these studies.

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Changes in Body Proportions Produced in Frog Embryos by Supra-Normal Temperatures.

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Several investigators have produced changes in body proportions by varying the conditions of temperature in which development occurs. Hoadley[1] has found that constant exposure to slightly supra-maximal temperatures produced gradations of microcephaly in *Rana pipiens* and *Rana sylvatica larvae*. Huxley[2] and his associates[3] and Gilchrist[4] have found that temperature *gradients,* within the ranges of temperatures normal to development, when properly applied to the developing amphibian egg for short intervals have a stimulative effect which is apparent in the development of the larva. Considering the fluid environment of the egg membranes and of the embryo itself, it is improbable that an embryo in nature would be subjected to a temperature gradient. However, short exposures of the whole egg to temperatures higher than the usual maximum for normal development must frequently occur. Possible long range effects on the developnig embryo of abnormal temperatures due to the heating of the spawning ponds of certain amphibia and to fever in the case of the pregnant mammal present problems in this connection. The present work was undertaken to test the effect on body proportions of short exposures of the whole amphibian egg and embryo to temperatures above the normal.

The eggs of *Hyla regilla,* the Pacific tree frog, were used in this work. An effort was made to obtain the larger clusters of 25 or

1 Hoadley, Leigh, *Growth,* 1938, **2**, 25.

2 Huxley, J. S., *Arch. F. Entwmech.,* 1927, **112**, 480.

3 Dean, I. L., Shaw, M. E., and Tazelaar, M. A., *Brit. J. Exp. Biol.,* 1928, **5**, 309.

4 Gilchrist, Francis G., *J. Exp. Zool.,* 1933, **66**, 15.

30 eggs each, for the eggs of a single cluster were the basis for each experimental series in order that all individuals of each series might be as nearly alike in age and genetic constitution as possible. Each cluster was divided into 5 equal groups. When the eggs of Group I reached the late blastula stage, they were exposed to a higher temperature in a thermostatically controlled water bath. After the exposure they were returned to room temperature. Group II was heated for the same length of time at the same higher temperature at the onset of gastrulation. Group III was similarly treated when in the yolk plug stage. In turn, Group IV was exposed at an early tail bud stage. All groups including the unheated controls (Group V) were allowed to develop at room temperature for 4 days at which time hatching usually began. Then all members of the series were removed from their membranes and preserved for study. Exposure periods of 2, 3, 4, 5, and 6 hr were tried at 10 temperature levels between 29° and 38°C. This report will consider the body proportions of 262 individuals representing 9 experimental series in which exposures were made to temperatures below 35°C. Above this temperature marked developmental abnormalities appeared. Careful measurements of the body proportions were made with an ocular micrometer in a dissecting microscope. As a check on the accuracy of the method, Series I consisting of 25 larvæ was remeasured without reference to the original measurements two months after the originals had been made. Differences between the two sets of data thus obtained were so slight as to convince the writer of the accuracy of the method.

TABLE I.

Effect of Heat Treatment on Body Proportions.

	Mean length		
	Heat treated group	Control group	Mean difference (± S.D.)
Head	2.005*	1.880	0.125† (± 0.028)
Body	6.128	6.166	0.038 (± 0.046)
Tail	6.727	6.784	0.057 (± 0.088)

*Ocular micrometer units.
† Highly significant: $P < 0.01$.

Statistical treatment of the data summarized in Table I shows a highly significant difference between the mean head length of control and experimental animals. Body length and tail length were apparently unaffected by the treatment. These results apply to all groups within the experimental series whether treatment was applied for 2 hr or for 6, whether at 29° or at 35°C. Similarly no significant differences among the groups of experimental animals were found to result from treatments applied at different stages of early embryonic development (from the early blastula stage through the tail bud stage). Therefore, in the final summary (Table I) these differences in treatment were disregarded, and all heat-treated embryos were considered as one group.

Since tail length and body length (defined as head length plus trunk length) comprise the total length, it is evident that the changed head length of the heat-treated animals has altered the ratio of body proportions without affecting the total length. This suggests that an age difference is not involved and that the proportions of the embryonic body may be altered by appropriate short exposures to higher temperatures without resorting to the use of temperature gradients.

Acknowledgment. Mr. John Y. Gilbert directed the statistical analysis of the data.

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Effect of Vitamin Deficiencies on Basal Metabolism and Respiratory Quotient in Rats.*

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It is now definitely established[1] that at least 3 of the vitamins in the B complex function in enzyme systems that control cellular respiration. One might expect, therefore, to find certain changes in the total metabolism of animals suffering from specific vitamin deficiencies. Early studies by Stare and Elvehjem[2] on the total oxygen uptake of tissues *in vitro* indicated that there was little difference in the rate of oxygen consumption between the tissues of normal and vitamin deficient animals. The more recent work which has demonstrated direct relationships such as thiamine to cocarboxylase, riboflavin to d-amino acid oxidase, xanthine oxidase, etc., and nicotinic acid to cozymase was carried out on individual enzyme systems. Thus it appears that any significant decrease in respiration is incompatible with life and that if a specific system is impaired the animal succumbs unless the energy can be obtained through other systems. Nevertheless, we felt that it was worth while to measure the basal metabolism and the respiratory quotient on rats suffering from uncomplicated B vitamin deficiencies. DuBois[3] has reviewed basal metabolism in disease, but as far as the authors are aware, no carefully controlled experiments have been carried out on animals having B vitamin deficiency diseases. In this paper we wish to report the results obtained in riboflavin, pyridoxine and pantothenic acid deficiencies. A total of 56 male rats have been used in these studies. The diets used for the production of each of the individual deficiencies are given in Table I.

The rations used for the production of riboflavin deficiency are based on the one described by Wagner, Axelrod, Lipton and Elvehjem.[4] Since Mannering, Lipton and Elvehjem[5] showed that a higher fat content of the diet produced a more severe riboflavin deficiency, the basal ration was modified to include varying amounts of fat. One-half of the animals were placed on the 4 modified basal rations and a similar number of rats was placed on these basal rations plus added amounts of riboflavin. The control rats received 12 γ of riboflavin per day.

The vitamin B_6 deficiency was produced on a diet described by Conger and Elvehjem.[6] The basal ration 613 was identical with the one described by the above workers and the other basal ration contained 60% of protein in place of the regular 18%. The control rats were fed 25 γ of vitamin B_6 per day.

The pantothenic acid deficiency was produced by feeding a synthetic ration containing all of the known B vitamins except pan-

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† Rockefeller Fellow. Present address, Sao Paulo Medical School, Sao Paulo, Brazil.

1 Elvehjem, C. A., and Wilson, P. W., *Respiratory Enzymes*, Burgess Publishing Co., Minneapolis, 1940; Green, D. E., *Advances in Enzymology*, Interscience Publishers, Inc., New York, 1941; Ball, E. G., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 253; Potter, V. R., *Medicine*, 1940, **19**, 441.

2 Stare, F. J., and Elvehjem, C. A., *Am. J. Physiol.*, 1933, **105**, 655.

3 DuBois, E. F., *J. Nutr.*, 1930, **3**, 331.

4 Wagner, J. R., Axelrod, A. E., Lipton, M. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1940, **136**, 357.

5 Mannering, G. J., Lipton, M. A., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 100.

6 Conger, T. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, **138**, 555.

TABLE I.
Composition of Rations.

	Riboflavin				Pyridoxine		Pantothenic acid	
	119	120	121	122	613	613A	789	789A
Dextrin	71	21.5	37.5	37.5				
Sucrose					75	33	73	
Cerelose								71
Casein (Labco)	18	18	18	18	18	60	18	20
Lard		22	14					
Crisco				14				
Butter fat	3	3	3	3				
Corn oil	2	2	2	2	3	3	5	5
Salts 4	4	4	4	4	4	4	4	4
Liver extract preparation	4	4	4	4				
Thiamine, γ	200	200	200	200	200	300	200	150
Riboflavin, γ					300	500	300	200
Nicotinic acid, mg	5	5	5	5	2.5	2.5	2.5	
Pyridoxine, γ	400	400	400	400			200	150
Pantothenic acid	500 γ	500 γ	500 γ	500 γ	2 mg	2 mg		
Choline, mg	100	100	100	100	100	200	100	100
Cod liver oil	2	2	2	2				

tothenic acid (Henderson, McIntire, Waisman and Elvehjem).[7] Two basal rations were used, one which contained sucrose and one in which glucose (cerelose) was the carbohydrate. The control animals received 100 or 500 γ of calcium pantothenate per day.

All the control animals received the particular vitamin by mouth. In the case of the riboflavin an alcoholic solution was used and the negative controls in this case were given an equivalent amount of alcohol without the riboflavin. All the animals received two drops of halibut liver oil by mouth per week. The basal metabolism determinations were made when the animals on the basal ration showed marked evidences of avitaminosis.

The animals were kept in an air-conditioned and humidity controlled room. One day before the BMR determinations were made the animals were removed to the room where the apparatus was kept and the food was removed from the cages. The determinations were made in a room kept at a constant temperature of 80°F.

The basal metabolism was determined in an apparatus made available to us by Dr. P. H. Phillips and Mr. Paul Boyer. This apparatus was patterned after that described by Schwabe and Griffith.[8] The technic used has been described by Schwabe, Emery, Griffith[9] and by Orsini.[10] Two or more determinations were made on each animal and only those results were used which showed good agreement in two consecutive runs. The BMR was expressed both as calories per square meter of body surface per 24 hr and as calories per kg body weight per 24 hr. The Diack[11] formula $SA = 7.42 \times W^{2/3}$ was used for calculation of the surface area.

The values for the BMR and RQ for all the animals are given in Table II.

Discussion. It is obvious that there are no significant differences in the BMR of the animals receiving the riboflavin deficient diets and those receiving the same diet plus riboflavin when the rate is expressed as calories per square meter. The values obtained are very similar to those which have been reported in the literature for normal rats. Definite differences are observed when the rate is based on the body weight of the animals. This difference is undoubtedly due

[7] Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutr.*, 1942, **23**, 47.

[8] Schwabe, E. L., and Griffith, F. R., Jr., *J. Nutr.*, 1938, **15**, 187.

[9] Schwabe, E. L., Emery, F. E., and Griffith, F. R., *J. Nutr.*, 1938, **15**, 199.

[10] Orsini, D., *An. Fac. Med. Univ. S. Paulo*, XVI (tones I), page 81, 1940.

[11] Diack, S. L., *J. Nutr.*, 1930, **3**, 289.

TABLE II.
Effect of Vitamin Deficiencies on B. M. R. and R. Q.

Ration	Supplement	Amt per day, γ	No. of rats	Weeks on experiment	Avg wt	B.M.R. Cal/M²/24 hrs	B.M.R. Cal/K/24 hrs	R.Q.
119	0		3	8	66	1,079	182	0.86
119	Riboflavin	12	3	8	197	1,126	149	0.84
120	0		3	8	57	1,073	214	1.10
120	Riboflavin	12	3	8	128	1,041	154	0.84
121	0		3	8	73	1,154	223	0.93
121	Riboflavin	12	3	8	150	1,107	156	0.84
122	0		3	8	53	1,041	196	1.17
122	Riboflavin	12	3	8	152	1,024	137	0.87
613	0		3	17	78	830	147	1.07
613	Pyridoxine	25	2	17	230	1,122	137	0.80
613A	0		6	10	62	773	146	0.91
613A	Pyridoxine	50	3	10	169	1,056	143	0.81
789	0		5	8	83	1,088	188	0.90
789	Ca pantothenate	500	2	8	123	1,154	164	0.82
789A	0		7	9	71	1,071	198	1.02
789A	Ca pantothenate	100	4	9	209	1,095	140	0.84

to the fact that the animals receiving riboflavin were much larger and contained more fatty tissue, which has little or no metabolism. The values obtained for the RQ of the deficient animals were higher in all cases than those for the control animals. It is interesting to note that the least difference was obtained on ration 119 which was low in fat and did not produce as severe riboflavin deficiency as the other rations.

The rats on the pyridoxine-deficient rations show a definite decrease in BMR when expressed on the basis of surface area. The fact that the BMR expressed on body weight is practically the same for the deficient and control rats lends greater importance to the decrease in BMR when expressed on surface area because one would expect that the lighter weight animal would have a disproportionately higher metabolism for each kg of tissue than the heavier animal. Expressed in another way, figures for BMR calculated on the basis of body weight normally show a slightly higher value for lighter animals. Since the values in pyridoxine-deficient animals (lower weight) are not higher than that of normal animals, the BMR is lowered even more than indicated by the figures expressed on the basis of surface area. The RQ is increased in pyridoxine deficiency.

Pantothenic acid-deficient rats showed a BMR identical with the control animals when expressed on the basis of surface area. In the case of body weight the same relationship was obtained as in the case of the riboflavin-deficient rats. The pantothenic acid-deficient rats showed an increased RQ similar to that observed in riboflavin and pyridoxine deficiency.

It is apparent that of the vitamins studied, pyridoxine is the only one that has a specific effect on BMR. However, in all the vitamin deficiencies studied there was produced a definite increase in RQ. One logical explanation for the increased RQ is one of incomplete combustion of the intermediate metabolites. These metabolites are either stored in the tissues or excreted as such thereby increasing the carbon dioxide-oxygen ratio. One may argue that the cause of this increase in RQ was due to inanition in all cases since it is well known that in inanition metabolites accumulate due to an insufficient supply of carbohydrate material. However, most of the animals did not show severe inanition, and most of them consumed almost as much food as the control animals when based on body weight. It is just as logical to suggest that the intermediate compounds accumulated because of faulty carbohydrate metabolism rather than insufficient supply of carbohydrates. Riboflavin deficiency is known to produce a disturbance in the respiratory mechanism, but up to the present time no re-

lationship has been demonstrated in the case of vitamin B_6 and pantothenic acid. Much more work is necessary before we can establish the true relationship between vitamin deficiencies and the respiratory quotient. But these preliminary experiments do indicate that studies along this line may be profitable and lead to a clearer understanding of the relation of vitamin deficiency to energy metabolism.

Summary. Riboflavin and pantothenic acid deficiencies in rats do not alter the BMR. A definite decrease in the BMR was observed in severe vitamin B_6 deficiency. All rats showing a riboflavin, vitamin B_6, and pantothenic acid deficiency had a higher RQ than normal rats. The possible explanation for this increased RQ is given.

13847 P

Production of a Diazotizable Substance by *Escherichia coli* during Sulfonamide Bacteriostasis.

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The classical paper of Woods,[1] describing the anti-sulfonamide action of p-aminobenzoic acid, crystallized the concept that sulfonamide bacteriostasis is the result of some interference with the normal metabolism of susceptible bacteria.[2] Woods[1] and Fildes[3] suggested that the p-aminophenyl grouping of the sulfonamides is sufficiently similar to that in the hypothesized metabolite, p-aminobenzoic acid, so that competitive inhibition occurs when the relative concentrations of PAB/Drug approach 1/5000. Our recent studies on the ionization of sulfonamides[4] seem to indicate that in terms of concentration of anions this ratio is 1/1 - 1/6.

Up to the present p-aminobenzoic acid has not been found essential for the normal growth of pathogenic bacteria nor has it been detected in such bacteria. During sulfonamide bacteriostasis, on the other hand, another diazotizable aromatic amine appears in the medium. The circumstances of its appearance are described in this preliminary note.

Synthetic medium* containing bacteriostatic concentrations of sulfonamide drugs[5] was placed in flasks or test tubes and inoculated with *Es. coli* (final dilution = 1 to 5 million per cc). Control samples taken at once were refrigerated and the remainder incubated at 37°C for 18 hr. Turbid cultures were centrifuged and diazo tests† were then done on the supernatant and the control samples. A typical result is as follows:

*Ammonium citrate	5	g
Magnesium sulfate	1.0	"
Sodium carbonate	3.0	"
Potassium dihydrogen phosphate	3.0	"
Sodium chloride	2.0	"
Iron ammonium citrate	.05	"
Nicotinic acid	.005	"
Glucose	.2	"
Acid hydrolysate of gelatin (Knight)	10	cc
Tap water to 1000 cc.		

1 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

2 (*a*) Mayer, R. L., *Bull. d. l'Acad. d. med.*, 1938, **117**, 727; (*b*) McIntosh, J., and Whitby, L., *Lancet*, 1939, **1**, 431; (*c*) Lockwood, J. S., *J. Immunol.*, 1938, **35**, 155; (*d*) Fox, C. L., Jr., *Am. J. Med. Sci.*, 1940, **199**, 487; (*e*) Mellon, R. R., Locke, A. P., and Shinn, L. E., *Am. J. Med. Sci.*, 1940, **199**, 749.

3 Fildes, P., *Lancet*, 1940, **1**, 955.

4 Fox, C. L., Jr., and Rose, H. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 142.

5 Rose, H. M., and Fox, C. L., Jr., *Science*, 1942, **95**, 412.

† The method of Bratton and Marshall was used.[6] This test is not specific for sulfonamides; positive results are obtained with most primary aromatic amino compounds.

Initial control diazo test	0.848 mg% sulfadiazine
After incubation diazo test	1.94
Increment	1.09 mg% diazotizable substance

Apparently significant amounts of diazotizable substances are produced during bacteriostasis. Control experiments showed that none is formed in the absence of either drug or bacteria. It is difficult to conceive of a chemical basis for intensification of the diazo color by the action of the bacteria on the drug itself. Nothing more can be said at present as to the source of the newly formed aromatic amine.

Obviously the first thought as to the identity of the new substance was p-aminobenzoic acid for this compound when diazotized and coupled yields as much color as an equimolar amount of sulfonamide. To investigate this possibility the new substance was obtained in sterile medium in this manner:

Synthetic medium containing bacteriostatic concentrations of sulfonamide drugs[5] was placed in cellophane bags‡ (ca 100 cc). In one series the sacs were tied off and immersed in 1 L. of the same drug medium in 3 liter Erlenmeyer flasks; in other series the sacs were fastened to 10 cm lengths of 5 mm glass tubing which passed through the cotton plugs in the necks of 1 L. florence flasks containing 800 cc of this medium. After autoclaving, the medium in the first series of Erlenmeyer flasks was inoculated (sacs sterile), but in the second series the sacs were inoculated through the glass tubes (medium in flasks sterile). After inoculation the concentration of bacteria was 1 to 5 million per cc. Both series with duplicate controls not inoculated were incubated at 37°C for 18 hr. Diazo tests[6] were then performed on samples of medium from inside and outside of the sacs. Typical results are shown in Table I.

To determine if the increase in azo color were the result of PAB production, the drug medium in the sterile sac was tubed, inoculated with *Es. coli,* and its bacteriostatic potency was gauged.[5] Were PAB production the cause of the large increase in azo color, the new drug medium from the sterile sac would not induce bacteriostasis. However, it did so, indicating that the substance responsible for the additional azo color does not block sulfonamide action as does PAB.

Experiments were then conducted to gauge the influence on the synthesis of this amine of (a) the concentration of drug, (b) the size of the inoculum, (c) the added presence of PAB or other sulfonamide inhibitors. In brief, the results were as follows: (a) very little diazotizable material was produced when concentrations of drug insufficient for

TABLE I.
Production of Diazotizable Substance During Bacteriostasis with 2.09 mg% Sulfadiazine.

Sample	Vol. of medium, cc	Diazo conc. after incubation, mg%*	Diazo total, mg*	Amt sulfadiazine in medium, mg	Increment of new amine, mg*
Sac—inoculated	92	3.01	2.86		
Flask—sterile	797	2.38	19.00		
		Total	21.86	18.2	3.6
Sac—sterile	100	2.68	2.68		
Flask—inoculated	1015	2.91	29.55		
		Total	32.23	23.0	9.0

(*Es. coli* 1-5 million per cc in gelatin hydrolysate medium.)
*For convenience in expressing the results the new amine is assumed to produce as much color as an equimolar amount of sulfadiazine.

‡ Thanks are due Dr. Otto Bier of Sao Paulo, Brazil, for the very helpful suggestion that bacteria can be grown in water with the nutrients in cellophane sacs.

[6] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537. Colors read in the Klett-Summerson photoelectric colorimeter.

bacteriostasis were used; excessive drug concentrations¶ did not augment its production, (b) the amount formed varied directly with the total number of bacterial divisions, (c) PAB reduced the production of chromogen in direct, linear proportion to the PAB/Drug ratio used; addition of infusion broth to this medium prevented its formation (and also bacteriostasis).

The production of this diazotizable substance is intimately associated with bacteriostasis; it is also formed with bacteriostatic concentrations of sulfapyridine and sulfathiazole but its detection is difficult with the less potent sulfanilamide which requires far greater concentrations¶ for bacteriostasis.[5]

Isolation of the new substance is in progress. It is stable to boiling, dialyzable, and extractable with butyl alcohol.

Summary and Conclusions. During growth of *Es. coli* in synthetic medium with bacteriostatic concentrations of sulfonamide, a diazotizable substance is produced. This is not p-aminobenzoic acid; in fact, its production is prevented by those amounts of PAB necessary to block the action of sulfonamides. The substance is not produced in the absence of sulfonamide nor when bacteriostasis does not occur.

During bacteriostasis by sulfonamides in this medium there is a change in the metabolism of *Es. coli* so that a metabolite with a diazotizable grouping similar to the therapeutically active group of the drug is produced. Experiments are in progress to determine whether the production of this substance is specifically elicited by the drug or whether it is a normal metabolite which accumulates when a sulfonamide interferes with the growth of the organisms.

¶ Colorimetric differences are not accurately detectable when high concentrations of drug are tested, since the errors may exceed the increment sought.

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New Crystalline Forms of Tomato Bushy Stunt Virus.

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From the first report on the crystallization of tobacco mosaic virus[1] until recently, only 2 additional crystalline forms of purified viruses have been reported, the non-birefringent dodecahedra[2,3] of tomato bushy stunt virus (TBS) and the faintly birefringent plates of tobacco necrosis virus.[4] These were obtained by means of crystallization with electrolytes such as ammonium sulfate. A new crystallization technic for large proteins employing hydrophilic colloidal materials such as heparin, starch, etc., has resulted in the crystallization of a hitherto uncrystallized hemocyanin, the usual crystalline form of the anisotropic viruses such as tobacco mosaic virus, and new crystalline forms of TBS[5] and of a tobacco necrosis virus.[6]

In continuing studies on the crystallization of TBS by means of heparin and other substances, two previously unobserved crystalline forms of this virus were obtained. One form was obtained using heparin under conditions somewhat different from those reported previously. To a solution of TBS in

* Fellow in the Medical Sciences of the National Research Council.

1 Stanley, W. M., *Science*, 1935, **81**, 644.

2 Bawden, F. C., and Pirie, N. W., *Brit. J. Exp. Path.*, 1938, **19**, 251.

3 Stanley, W. M., *J. Biol. Chem.*, 1940, **135**, 437.

4 Pirie, N. W., Smith, K. M., Spooner, E. T. C., and McClement, W. D., *Parasitology*, 1938, **30**, 543.

5 Cohen, S. S., *J. Biol. Chem.*, 1942, **144**, 353.

6 Cohen, S. S., and Stanley, W. M., unpublished data.

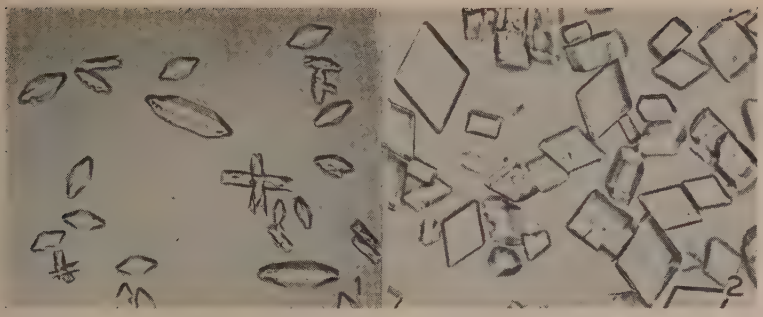

FIG. 1. FIG. 2.

FIG. 1.
Tomato bushy stunt virus crystallized by means of sodium heparinate. × 239.
FIG. 2.
Tomato bushy stunt virus crystallized by means of sodium sulfonate of polyanethole (Liquoide Roche). × 534.

water at a concentration of 4.0 mg per cc the solid sodium heparinate† was slowly added at room temperature and dissolved with rubbing until an opalescence due to protein precipitation was observed. This appeared at a concentration of about 5% with respect to heparin. The mixture was cooled in an ice-bath until the amorphous precipitate completely redissolved, and was then stored at 4° overnight. Well defined crystals, some of which were 0.2 mm in length, appeared in that time. Warming this mixture to room temperature and rechilling resulted in a new larger crop of smaller crystals of the same shape. The new non-birefringent crystals, shown in Fig. 1, appear to be eight-sided prisms whose ends are tapered by joint triangular planes. After being washed several times with 3 M ammonium sulfate, the crystals were dissolved in water and analyzed for carbohydrate and phosphorus as described previously.[5] There was no evidence of combination of virus with heparin from the carbohydrate to phosphorus ratio. The sedimentation constant of the dissolved crystals was normal for TBS,[7] $S_{20} = 129 \times 10^{-13}$. This was estimated at 3 mg per cc in 0.1 M borate buffer at pH 7.1 by Dr. M. A. Lauffer of this laboratory. The redissolved crystals showed no significant change of virus activity as estimated on *Nicotiana glutinosa* L., and on cowpea.

The search for other effective crystallizing agents resulted in the testing of Liquoide Roche, the sodium sulfonate of polymerized anethole. Under conditions comparable to those just described, but at a Liquoide concentration of approximately 4%, another non-birefringent crystalline form appeared. The rhombohedral crystals are shown in Fig. 2.

The property of possessing numerous crystalline forms under varying conditions is common to many of the simpler proteins, such as edestin, insulin, ribonuclease, etc. This property of tomato bushy stunt virus once again emphasizes its relationship to substances which are expected to behave according to chemical and physical laws. It can be expected that an examination of these various well shaped forms by X-ray technic should yield considerable information concerning certain intrinsic properties of this virus molecule.

† The writer wishes to express his thanks to the Roche-Organon, Inc., for gifts of sodium heparinate.

7 Lauffer, M. A., *J. Physic. Chem.*, 1940, **49**, 1137.

13849

Effect of *Lactobacillus casei* ϵ Eluate Fraction on Reproduction in the Domestic Fowl.

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Hutchings, Bohonos, Hegsted, Elvehjem and Peterson[1] have reported that a norite eluate fraction required by *Lactobacillus casei* ϵ is essential for the normal growth of chickens. In further studies Mills, Briggs, Elvehjem, and Hart[2] found that the eluate fraction is essential for hemoglobin formation and normal feathering in young chicks. Schumacher and Heuser[3] have reported that mature hens require a factor, termed the alcohol-precipitate factor, for egg production, hatchability and maintenance of body weight. The possible relation of the *Lactobacillus casei* ϵ factor and the alcohol-precipitate factor, as well as other chick factors, is suggested by Mills, *et al.*[2]

In studies on the effect of various members of the vitamin B complex on reproduction in poultry we have observed that a norite eluate of solubilized liver extract prepared by a modification of the method of Hutchings, Bohonos and Peterson[4] contains a factor or factors essential for normal embryonic development in the domestic fowl. The results of these studies are reported here.

Single Comb White Leghorn Pullets were housed in individual laying cages and artificially inseminated. Eggs were incubated weekly. In the first experiment the basal ration had the following composition: Sucrose, 60; Casein,† 18; gelatin, 5; salts IV,[5] 5; soybean oil, 5; whey concentrate, 5; and fish oil, 2 (3000 I. U. vitamin A, 400 A.O.A.C. units of D per g). Each kg of ration also contained thiamin, 3 mg;‡ riboflavin, 5 mg; calcium pantothenate, 15 mg; pyridoxin, 8 mg; nicotinic acid, 100 mg; and choline, 2 g. Oyster shell was supplied *ad libitum*. After the birds had been on the basal ration for 7 weeks, 3% kidney residue replaced the whey concentrate as a source of biotin. The kidney residue used in these rations was prepared by extracting dried defatted kidney with large volumes of hot water and 50% ethanol. Dextrin was substituted for the sucrose during the tenth week.

After 9 weeks on the basal ration hatchability was at zero. The birds were continued on the basal ration for an additional 3 week depletion period, then supplements were added as shown in Table I. Since the inositol content of the basal ration was unknown one hen was given 0.2% inositol. One hen was fed the norite eluate fraction of solubilized liver extract, 2 birds were given 0.2% inositol plus the norite eluate fraction and 2 birds were given 4% solubilized liver extract. The results are given in Table I.

The results of this experiment furnish evidence that both solubilized liver extract and a norite eluate fraction of solubilized liver extract contain a factor or factors essential for normal development of the

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1 Hutchings, B. L., Bohonos, N., Hegsted, D. M., Elvehjem, C. A., and Peterson, W. H. *J. Biol. Chem.*, 1941, **140**, 681.

2 Mills, R. C., Briggs, G. M., Elvehjem, C. A., and Hart, E. B., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 186.

3 Schumacher, A. E., and Heuser, G. F., *Poul. Sci.*, 1940, **19**, 315.

4 Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

† Borden's H.5P.

5 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 458.

‡ We are indebted to Borden and Company, Special Products Division, New York City, for the fish oil and whey concentrate; to Merck and Company, Rahway, N.J., for the crystalline vitamins; and to Wilson Laboratories, Chicago, Ill., for the solubilized liver extract and gelatin.

TABLE I.
Supplements to Basal Ration and Percent Hatch of Fertile Eggs.

	Group and supplements to the basal ration							
	1		2		3		4	
	0.2% inositol		Norite eluate equivalent to 10% solubilized liver extr.		0.2% inositol + norite eluate equivalent to 10% solubilized liver extr.		4% solubilized liver extr.	
Week	Fertile Eggs	% Hatch	Fertile Eggs	% Hatch	Fertile Eggs	% Hatch	Fertile Eggs	% Hatch
12	11	0.0	0	—	18	0.0	0	—
14	8	0.0	1	0.0	17	23.5	0	—
16	10	0.0	4	100.0	20	70.0	0	—
18	8	0.0	0	—	20	75.0	2	100.0
20	10	0.0*	0	—†	20	75.0‡	8	87.5
22	10	60.0	0	—	17	70.6	4	100.0
24	8	75.0	0	—	21	47.6	18	83.4
26	9	88.9	1	100.0	16	31.2	15	80.0
28	5	80.0	7	85.7	19	5.3	19	57.9
30	6	100.0	10	80.0	19	5.3	19	94.6

*Norite eluate equivalent to 10% solubilized liver extract added.
†0.2% inositol added.
‡Norite eluate fraction discontinued.

chicken embryo. The bird receiving only the norite eluate came into production 3 weeks after the supplement was added then stopped laying until 4 weeks after the inositol was added. Obviously with only one bird no final conclusions can be drawn. The bird receiving inositol alone continued to lay but all her eggs failed to hatch until the norite eluate was added. The response to the latter supplement was almost immediate.

The birds receiving only the solubilized liver extract responded very slowly because they were in such poor condition at the time the supplements were added. They each

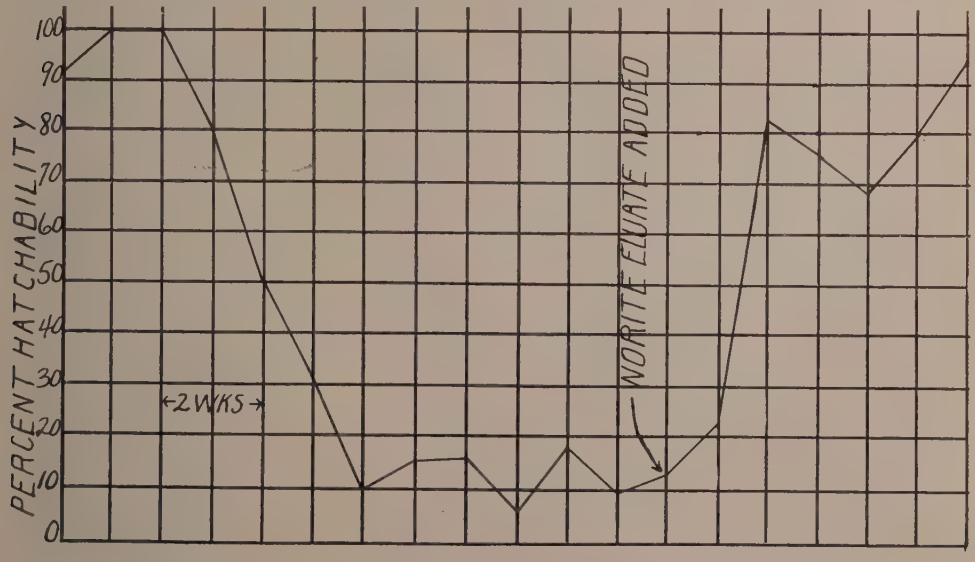

FIG. 1.
Hatchability of Fertile Eggs.

weighed 950 g when the supplement was added and at the time they started laying again their weight had increased to approximately 2 kg each.

The basal ration used in the second experiment was identical to that used in the first except that 0.1% inositol was added.

In this experiment 4 birds were fed the basal diet. Their average hatchability dropped from above 90% to less than 20% in 6 weeks. They were continued on the basal diet for 6 weeks longer to insure thorough depletion and then were fed the norite eluate fraction preparation equivalent to 10% solubilized liver extract. The results are given in Fig. 1.

The results of this experiment are in agreement with the results of the first experiment and show conclusively that the norite eluate of solubilized liver extract contains a factor or factors essential for normal development of the chicken embryo.

A deficiency of this factor resulted in a less pronounced decline in egg production than in hatchability. Some birds continued to lay when fed the basal ration while others would go out of production and lose weight. Such results suggest that the basal ration contained a suboptimal amount of the factor involved. Further studies are in progress to clarify this point.

Summary. Evidence is presented to show that the *Lactobacillus casei* ϵ eluate fraction is essential for normal development of the chicken embryo.

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13850 P

Chemotherapy in *Cl. welchii* Infection in Mice.

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The importance of *Cl. welchii* infection in war wounds has given impetus to work in this field during the past year. The early literature on the subject has been well reviewed by Bliss, Long and Smith.[1] Recently, Sewell, Dowdy and Vincent[2] have been able to produce an infection in dogs which closely simulated human infection. They found that sulfadiazine therapy gave the best results.

In this study, a comparison of the efficacy of sulfanilamide, sulfapyridine, sulfathiazole and sulfadiazine‡ in experimental *Cl. welchii* infection in mice has been made. By feeding the animals a moist cooked diet in which the drugs had been incorporated, it was possible to maintain blood concentrations comparable to those used in the treatment of patients ill with serious infections.

The drug-diet was prepared in the following manner: The required amount of drug was dissolved in 1000 ml of water at 100°C. To this was added slowly 280 g of a modified Sherman dry diet and the mixture cooked with stirring until it was the consistency of thick mush. It was then allowed to cool, weighed and the amount of drug calculated as mg per g of food. The per cent of drug

* A. B. Kuppenheimer Foundation.

† Joseph B. DeLee Fellow 1941.

1 Bliss, E. A., Long, P. H., and Smith, D., *War Med.*, 1941, **1**, 799.

2 Sewell, R. L., Dowdy, A. H., and Vincent, J. G., *Surg., Gyn. and Obst.*, 1942, **74**, 361.

‡ The sulfonamides used in this study were furnished through the courtesy of Lederle Laboratories, Inc. (sulfadiazine), Merck and Company (sulfapyridine), and the Department of Medical Research, Winthrop Chemical Company (sulfanilamide and sulfathiazole).

TABLE I.
Comparison of Drug Ingested and Blood Concentrations.

Drug-diet	Drug ingested: Mg/mouse/day Mean ± S.E.	Drug ingested: G/kg/day Mean ± S.E.	Blood concentrations mg/100 Mean ± S.E.
2.0% sulfanilamide	41.6 ± 2.68	2.18 ± .26	16.0 ± 1.15
1.5% sulfapyridine	36.1 ± 2.20	1.99 ± .16	12.5 ± 2.04
2.0% sulfathiazole	37.6 ± 2.84	1.89 ± .05	11.0 ± 0.82
0.4% sulfadiazine	11.1 ± 0.74	0.61 ± .03	12.5 ± 1.48

used was calculated on the basis of the dry meal, *i.e.*, for a 1% drug-diet 2.8 g of drug was used, for a 2% diet 5.6 g, etc. The amounts correspond to roughly 0.2 and 0.4% concentrations of the entire moist diet. Sulfadiazine was too insoluble to dissolve completely in water, so the drug was thoroughly mixed with the dry food and then added to the water.

Table I presents the average amounts of drug ingested on the preferred diets, namely, 2% sulfanilamide, 1.5% sulfapyridine, 2.0% sulfathiazole, and 0.4% sulfadiazine. The corresponding mean blood concentrations obtained at the time of inoculation which was 4 days after drug had been started are given also. Since equipment for micro determination of drug concentration was not available, it was necessary to kill the animals used for each blood determination. Blood was drawn for these determinations at the same time of day that inoculations were always made, 3-5 P.M. The mean drug concentration in the blood and the corresponding drug intake per day ± standard error have been calculated from determinations made on groups of 15-20 animals. The mice (15-22 g) were kept in individual cages and the food cups were weighed daily.§

The animals were inoculated intramuscularly in the inner aspect of the thigh 4 days after drug feeding had begun, and the drug was continued 7 and 14 days after inoculation.

The strain of *Cl. welchii* used was one which had been isolated from a patient who had a fatal postpartum infection. Inoculation in mice regularly produced a generalized infection accompanied by necrosis of the thigh muscle at the site of inoculation. An almost uniform virulence for mice was obtained over a period of several months by the following standardization of technic: The original isolation culture was kept in the refrigerator in Rosenow's brain broth. Each culture used for inoculation was prepared by starting with a transfer from this original broth. Transfers were then made daily for 7 days. The last transfer was made into a tube containing brain and 9 cc of broth. The 18-hr growth from this tube was diluted 1:4 with physiological saline and 0.25 ml (0.0625 ml undiluted culture) was inoculated into the thigh muscle. An attempt was made to use an inoculum sufficient to kill approximately 80% of the untreated animals. After the dose had been determined it was further controlled by the inoculation of 10 control animals with each series of drug-fed animals.

The results reported here are the combination of experiments which yielded very similar results on repeated trials. As can be seen, sulfanilamide was of little or no value, sulfapyridine was somewhat better, but results were not consistent, sulfathiazole gave good results, but sulfadiazine was the best.

These results are considerably better than those reported in a similar study made by Bliss, Long and Smith.[1] It is quite possible

TABLE II.
Comparison of Sulfonamides in *Cl. Welchii* Infection.

Diet	No. of animals	% survivals
Untreated controls	75	15.7
2% sulfanilamide	44	27.2
1.5% sulfapyridine	48	46.4
2% sulfathiazole	54	79.6
0.4% sulfadiazine	46	93.4

§ We are indebted to Dr. R. N. Bieter for full information on the preparation of cages and food cups.

that this difference is due to the fact that we produced drug concentrations of 11-19 mg % in the blood of our animals whereas they produced concentrations of only 5-7 mg %.

Although the animals were given drug before inoculation, the experiments cannot be considered entirely from the standpoint of prophylaxis, because all the animals showed a generalized infection 6-18 hr after inoculation, some of them developed hematuria, and all of them stopped eating or ate very little food for a day or two. If they survived this period, they usually remained well throughout the following 2-week observation period.

Even though the animals survived, the tissue at the site of inoculation became necrotic and sloughed off. It made very little difference whether the drug was continued 7 or 14 days after inoculation, the lesions though walled off, did not heal. Vegetative organisms could be observed in direct smear and *Cl. welchii* were isolated in culture.

Conclusions. 1. Mice inoculated intramuscularly with *Cl. welchii* and treated orally with sulfonamides responded best to sulfadiazine (sulfadiazine 93.4%, sulfathiazole 79.6%, sulfapyridine 46.4%, and sulfanilamide 13.8% survival).

13851

Comparison of the Activity and Distribution of Iodine, in Reptilian and Mammalian Thyroids.

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The interesting observation that turtle or alligator thyroid had practically no effect on the basal metabolic rate of man was made by Swingle and Martin.[1] The iodine content of the thyroid preparations fed was very high, over 4 mg per g, and so, by mammalian standards, these glands should have been fully iodized and resting, with alveoli filled with dense colloid. Such thyroids have always been found to have strong physiological activity. In contrast to the action of these preparations on man, when they were given to rabbits, the metabolic rate was raised to about the same extent as when cattle thyroid was fed. Swingle and Martin attribute the different behavior of reptilian thyroid in man and the rabbit to the fact that the dose used for rabbits was relatively large.

Since this work was reported, methods for the accurate estimation of thyroxine in thyroid have been developed and these were applied to various samples of reptile thyroid, including those used by Swingle and Martin, to determine whether the distribution of iodine differed from that found in mammalian thyroid. As a check on the validity of the analyses, the metamorphosing action of these preparations on tadpoles was studied and compared with the action of several dogs' thyroids.

Methods. Thyroxin was measured by Blau's[2] modification of the Leland and Foster[3] method. In addition to the dried alligator and turtle thyroids used by Swingle and Martin, samples of which had been in this laboratory since Marine determined their influence on rabbit metabolism in 1926, we obtained thyroids of 4 Caribbean Sea turtles. These animals weighed between 70 and 110 kg and had been kept in a warm room on their backs for about 10 days before they were killed. The thyroids seemed somewhat edematous, an observation that is supported by the high water content found on drying the tissue in vacuum desiccators over BaO. A small piece of each specimen was fixed in formalin for microscopic examination.

[1] Swingle, W. W., and Martin, K. A., *J. Exp. Zool.*, 1926-7, **46**, 277.

[2] Blau, N. F., *J. Biol. Chem.*, 1935, **110**, 351.

[3] Leland, J. P., and Foster, G. L., *J. Biol. Chem.*, 1932, **95**, 165.

TABLE I.
I Distribution in Reptile Thyroid.

Specimen	Water, %	Thyroxine I, mg per g	Total I, mg per g	Thyroxine I: Total I %	Thyroid epithelium
Turtle 2	83.6	0.95	2.63	36.2	Low cuboidal
" 3	77.1	0.93	2.43	38.3	Cuboidal
" 4	81.0	0.90	2.40	37.5	Low cuboidal
" 5	79.2	1.34	3.84	34.9	Flat "
" S & W		1.48	4.62	31.9	" "
Alligator S & W		1.49	4.18	35.6	" "
Dog 6		0.67	2.32	28.9	Cuboidal
" 5		1.19	3.94	30.2	Flat cuboidal
" 2		1.38	4.08	33.8	" "

Results. The observations are recorded in Table I, together with those of a few dogs' thyroids of similar I and thyroxine content.

Thyroxine and total I values of all the reptile thyroids as shown in Table I are very high, turtle 5, S and W and the alligator specimens being among the highest that have been recorded in the literature. Corresponding to the high thyroxine and I, all the glands show, as one would expect, a very high degree of involution, *i.e.,* uniform dense colloid and flat cuboidal epithelium.

Influence on metamorphosis. To dishes containing 5 tadpoles, each, in 250 cc of tap water, single 1 mg and 2 mg doses of powdered reptilian thyroid were added. For comparison, other dishes, each containing 5 tadpoles in 250 cc of tap water, were set up and 1 and 2 mg doses of dog thyroids of high I and thyroxine content added. The dried thyroids were powdered, passed through fine silk bolting cloth and weighed on a microbalance. Control dishes with no thyroid were also included. The tadpoles were fed on liver for 2-3 hr daily. The single 1 or 2 mg dose of thyroid was added after removing the liver and changing the water and was left for 24 hr. Thereafter the tadpoles were fed daily for 2 or 3 hr on liver only. In 5 days great shrinkage in size and almost complete metamorphosis had taken place with 2 mg of the preparations having the highest I and thyroxine values, as shown in Table II. The rate of metamorphosis varied directly with the I and thyroxine content of the glands. With 1 mg doses more than twice the period elapsed before the same stage was reached. But here, too, the rate of metamorphosis varied directly with the thyroxine content of the preparations.

From these observations on the thyroxine content and tadpole metamorphosis, no evidence was obtained that would indicate any difference in the activity of reptilian and mammalian thyroids that contain similar amounts of iodine and thyroxine.

TABLE II.
The Influence of Reptile Thyroid on Metamorphosis.

Specimen	1st day	2nd day	3rd day	4th day	5th day	Total I	Thyroxine I
Turtle 3	0	0	±	+	++	2.43	0.93
" 4	0	0	+	+	++	2.40	0.90
" 5	±	±	+	+	++	3.84	1.34
" S & W	0	±	+++	++++	++++	4.62	1.48
Alligator	0	±	+++	+++	++++	4.18	1.49
Dog 127	0	0	+	+++	++++	3.54	0.98
" 1	0	±	+	+	++	1.63	0.51
Control	0	0	0	0	0		

0 = no change or very slight shrinkage back of gills.
± = slight shrinkage back of gills.
+ = slight kite shape, moderate shrinkage back of gills.
+++ = moderate kite shape, marked shrinkage back of gills.
++++ =marked kite shape, nearly complete tail absorption.

13852

Lack of Effect of Growth Hormone on Deposition of Radiostrontium in Bone.*

WALTER MARX AND WILLIAM O. REINHARDT. (Introduced by Herbert M. Evans.)
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The pituitary growth hormone is known to play an important role in the regulation of processes concerned with bone formation. Deposition of minerals in the skeletal system represents one phase of this complex phenomenon. The experiments reported here were carried out in order to determine whether mineral deposition in bone is influenced by growth hormone. Pecher, in experiments on the deposition of radioactive isotopes of calcium and strontium, has shown that the distribution of these two elements in body tissues is similar.[1] In the present experiments, radiostrontium was employed, since it is more easily available and the methods for its measurement are more practical.

In order to exclude possible effects of other pituitary factors, hypophysectomized rats were used as test animals. Female rats were hypophysectomized at 26-28 days of age. In each experiment, the rats, after a post-operative interval that varied in different experiments from 10-20 days, were divided into two groups, one of which received daily intraperitoneal injections of growth hormone, the other group serving as control. 2-5 days after onset of the growth hormone treatment, radioactive strontium was given intraperitoneally to both the experimental and control groups. At varying intervals (8-48 hr) after the strontium administration, the animals were autopsied. Details of the experimental procedures are given in Table I. At autopsy, femurs and mandibles (including teeth) were freed from soft tissues, weighed and ashed (with HNO_3, followed by dry ashing in a muffle furnace). The radioactivity of each specimen was determined using the Lauritzen electroscope. Appropriate standards of radiostrontium were used for estimating the quantities of strontium deposited. Correction for self-absorption did not affect the results within the limits of error of the method.

The growth hormone preparations were "cysteine treated globulin fractions."[2] The preparations used in Experiments I, II, IV and V were contaminated with less than 1% of any of the 5 other "accepted" pituitary hormones; in the preparation used in Experiments III and VI no interstitial cell stimulating or adrenocorticotropic hormones were detected at a total dose of 3.0 mg, whereas a trace of thyrotropic activity was demonstrable at this level (4-day test in hypophysectomized rats). In none of the preparations used was lactogenic hormone found at a level of 10 mg (systemic crop test in squabs). The strontium lactate preparations employed contained radioactive strontium (Sr^{89}, half life 55 days).[4] Radioactivity of the preparations varied from 3 to 7 microcuries per mg (1 to 6 microcuries per total dose). The quantities of growth hormone and strontium given, and the duration of the injections are indicated in Table I.

Table II summarizes 6 experiments in which a total of 43 rats was used. In 5 out of 6 experiments, there was practically no

* Aided by grants from the Board of Research of the University of California and the National Research Council Committee on Research in Endocrinology.

1 Pecher, C., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 86.

2 Fraenkel-Conrat, H. L., Meamber, D. L., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1940, **27**, 605. (A modification of the procedure will be published in the near future.)

3 Marx, W., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1942, **30**, 1.

4 Stewart, D. W., Lawson, J. L., and Cork, J. M., *Phys. Rev.*, 1937, **52**, 901.

TABLE I.
Experimental Conditions.

Exper.	No. of hyp. rats		Growth hormone		Radiostrontium	
	Exp.	Contr.	Total dose, mg*	Period of injection, days	Total dose, mg	Period of injection, days
I	3	3	0.6	3	1.0	1
II	3	3	1.2	4	1.0	1
III	4	4	1.5	5	0.5	1
IV	3	4	1.8	3	0.3	1
V	4	4	2.0	8	0.5	4
VI	4	4	3.25	15	2.25	10

*The average potency of the growth hormone preparations varied from 30-50 growth hormone units per mg (10-day test in hypophysectomized rats).[3]

TABLE II.
Effect of Growth Hormone on Deposition of Radioactive Strontium in Bone.

Exper.	Group	Avg body wt gain in g	Strontium content in μg/100 mg ash			
			Femur		Mandible	
			Avg values	Range of values individual rats	Avg values	Range of values individual rats
I	Exper.*	5	32	27-38	21	17-25
	Contr.	0	31	30-31	18	17-19
II	Exper.*	9	40	37-42	25	24-27
	Contr.	—2	29	23-36	17	12-23
III	Exper.*	15	18	16-22	12	10-13
	Contr.	4	20	19-22	12	12-13
IV	Exper.*	4	14	12-14	—	—
	Contr.	—2	13	12-14	—	—
V	Exper.*	16	20	17-25	14	12-16
	Contr.	1	19	17-22	13	11-14
VI	Exper.*	20	75	65-85	—	—
	Contr.	—3	73	60-82	—	—

*Experimental groups received growth hormone in the amounts specified in Table I.

difference between growth hormone treated and untreated animals; the average values for the strontium content of femur and mandible were essentially the same, as were the ranges covered by the values for individual animals. The results of one of the 6 experiments might indicate some difference in the strontium depositions of treated and of control animals (Experiment II). This difference, however, was not considered significant, since a repetition of this experiment did not confirm these results (Experiment III). The quantity of strontium deposited in femur or mandible in the various groups was quite constant when calculated as per cent of the total amount injected, regardless of growth hormone administration, the amount of strontium injected, or within the limits of these experiments, the duration of treatment. The average values for the percentage deposited of the total strontium injected were femurs, 2.6% (range 2.0-2.9%); mandibles, 2.3% (range 1.8-2.7%).

It appears, therefore, in experiments of relatively short duration, that untreated hypophysectomized rats, in which growth stasis had been reached, deposited essentially the same amount of strontium in bone, as did rapidly growing animals treated with growth hormone. These results would appear to indicate that mineral deposition in bone as indicated by strontium metabolism is a reaction which, in these acute experiments, can proceed independently of the action of the

pituitary growth hormone, a principle which is generally assumed to control somatic growth. In agreement with this finding are observations by various authors that mineral exchange in the skeletal system may continue even after practical cessation of growth, such as is the case in old age, or, in young animals, after ablation of the pituitary.

Summary. Hypophysectomized rats treated with growth hormone and consequently in a state of rapid growth, deposited in femur and mandible, when injected with radioactive strontium, essentially the same amounts of strontium, as did untreated hypophysectomized control rats injected simultaneously with the same amount of radioactive strontium.

We wish to express our gratitude to Dr. John H. Lawrence and the staff of the Crocker Radiation Laboratory of the University of California, for their generosity in supplying the radioactive strontium used in these experiments.

13853 P

Purification of Antigonadotropic Sera by Enzymatic Digestion.*

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It has been shown by Coghill *et al.*[1] that antitoxin in horse serum can be purified to a considerable extent without much loss of activity by digestion with the enzyme Taka-Diastase. Since the antitoxins as well as the antigonadotropic substances produced in the blood of animals over a long period with gonadotropic extracts are found in the globulin fraction of the serum, it was considered probable that the antigonadotropic serum could be purified by the same method. The results of our preliminary work support this view.

Antigonadotropic serum of the horse and the goat were used in this study. The horse serum was obtained from a mare pony which had been injected 3 to 6 times a week for 3 years with a crude sheep pituitary extract (SAP). The goat serum was obtained from an adult female goat which had been injected 5 to 6 times a week for 2 years with a purified pregnancy urine preparation (PU).

Both sera were digested with Taka-Diastase (obtained from Parke, Davis and Company, Detroit, Michigan) in a manner similar to that employed by Coghill *et al.*[1] One volume of serum was diluted with 2 volumes of distilled water and the pH adjusted to 4 by the addition of crystalline citric acid. Taka-Diastase in the proportion of 5 mg to each ml of undiluted serum was added together with a few drops of toluene. The mixture was shaken well and incubated for 96 hr at 37°C. During the digestion a large, flocculent precipitate was formed. At the end of 96 hr the mixture was filtered. The precipitate was removed from the filter paper, taken up in a volume of distilled water equal to that of the undiluted serum, and shaken well with glass beads to make a homogeneous suspension. This was filtered as before. The combined filtrates were dialyzed first against running tap water and then against several changes of distilled water.

This practically salt-free solution was made 40% saturated with ammonium sulfate and allowed to stand in the refrigerator for several hours. The precipitate (Fraction A) was removed by filtration through a hard filter paper (S&S 575). The filtrate was made 50% saturated with ammonium sulfate and, after standing, the precipitate (Fraction B) was removed as before. Assays showed that the antigonadotropic substance was present in fraction A of the goat serum, and

* Supported in part by a grant from the Wisconsin Alumni Research Foundation and from Hospital Liquids, Inc., Chicago, Ill.

1 Coghill, R. D., Fell, N., Creighton, M., and Brown, G., *J. Immun.*, 1939, **39**, 207.

TABLE I.
Solids Content and Antigonadotropic Activity of Digested Horse and Goat Sera.

Serum preparation	Solids mg per ml	Solids %	Serum ml eq	Dose gonadotropin	Ovarian weights, mg	No. rats
Horse antiserum untreated	82.7	100	1.0	100 mg eq SAP	13	6
Precipitate*	21.3	25.7	1.0	" " " "	61	4
Filtrate*	38.5	46.5	1.0	" " " "	16	6
Fraction A	12.2	14.7	1.0	" " " "	59	6
Fraction B	19.9	24.0	1.0	" " " "	15	6
Sub-fraction B	9.4	11.3	1.0	" " " "	13	6
Goat antiserum untreated	107.2	100	0.75	0.5 mg PU	17	3
Precipitate*	39.0	35.6	1.0	" " "	62	3
Filtrate*	40.0	37.3	1.0	" " "	13	5
Fraction A	3.4	3.1	0.25	" " "	17	9
Fraction B	6.4	5.9	0.75	" " "	59	3

*After digestion.

in fraction B of the horse serum. Further fractionation of the latter was accomplished by chilling to 0°C (after dialysis to remove excess salt) and adding acetone at —5°C to make a 40% acetone solution. After several hours standing in the cold the mixture was centrifuged. The supernatant liquid was poured off and the bottles were inverted to drain off the acetone. The acetone adhering to the precipitate was removed by inserting a 2-hole rubber stopper into the centrifuge bottle and by removing the air by way of one opening while dust-free air was admitted by way of the other. Thus the precipitate was largely freed of acetone and at the same time it was dissolved in the residual water (Sub-fraction B).

For purposes of determining the antigonadotropic activity of the serum fractions, 21-day-old female rats were used as assay animals. Appropriate doses of the horse serum fractions and the sheep pituitary extracts were injected subcutaneously and separately on opposite sides of the body. Each goat serum fraction was mixed with PU and administered in one injection. Both groups of rats were injected twice a day for 4.5 days. They were killed on the morning of the 6th day and the ovaries were removed and weighed. A representative sample of the results is given in Table I.

The data presented indicate that digestion of antigonadotropic serum with Taka-Diastase for 96 hr removed approximately one-half the solids and little, if any, of the antigonadotropic activity. Fractionation with precipitating agents resulted in the removal of more solids. The antigonadotropic substance of the horse serum was concentrated in a fraction which represents about 12% of the original solids, while that of the goat serum was concentrated in a fraction which represents 3.1% of the original solids.

13854

Growth of Platyfish (*Platypoecilus maculatus*) Free from Bacteria and Other Microörganisms.

James A. Baker and M. S. Ferguson. (Introduced by C. TenBroeck.)

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Since 1885 when Pasteur[1] made the statement that no life was possible in vertebrates without intestinal bacteria, there have been sporadic attempts to grow various animals in an environment free from microörganisms. In the early days, these experiments were conducted largely to prove or disprove this point. Schottelius[2] working with chicks, O. Metchnikoff[3] working with frog tadpoles, and Moro[4] working with toad tadpoles, were unable to obtain appreciable growth in their axenic* vertebrates and concluded that an intestinal flora was necessary for the development of these animals. On the other hand, Nuttall and Thierfelder,[5] Kuster (see Wollman), Cohendy,[6] and Wollman[7] were convinced that bacteria are not necessary in view of their experiments with young guinea pigs, a young goat, chicks, and frog tadpoles, respectively. Both Cohendy and Wollman found their axenic animals would live for weeks and that the growth paralleled that of controls. Wollman observed that failure to obtain growth in axenic animals might be due to the experimental procedures, as for instance the high temperatures at which food was sometimes autoclaved—a factor which is now known to be very important.

During the last decade, Reyniers and his associates at the University of Notre Dame[8] have had outstanding success in keeping axenic guinea pigs and chickens until they were several months of age. By employing special technics and equipment, together with carefully prepared and supplemented diets, they have been able to bring these animals to apparent physical maturity. As yet no offspring have been obtained in an environment free from microörganisms. Reproduction in an axenic vertebrate would indicate that the animals are physiologically normal, and multiplication through several generations would be definite proof that among higher animals life is possible without microörganisms.

Many invertebrate animals, both free-living and parasitic—particularly certain protozoa, nematodes, and insects—have been reared through successive generations in media free from microörganisms (See Trager's[9] review). The cultivation of these animals has required special diets and culture

1 Pasteur, L., *Compt. Rend. Acad. Sci.*, 1885, 100.

2 Schottelius, Max, *Arch. f. Hygiene*, 1899, **34**, 210; *Ibid.*, 1902, **42**, 48; *Ibid.*, 1903, **67**, 177.

3 Metchnikoff, O., *Ann. de l'Inst. Pasteur*, 1901, **15**, 631.

4 Moro, *Jahrbuch für Kinderheilkunde*, 1905, **2**, 467.

* The adjective *axenic* is introduced to denote a living organism that is free from all other demonstrable organisms. It is a more convenient term than expressions currently used, *e.g.*, "animals free from microörganisms," "animals free from contaminating organisms," and "animals free from contaminants," and in meaning it is more correct than the adjectives *sterile* and *germ-free*. *Axenic* is derived from two Greek words: *A*—meaning *without* or *free from*, and *Xenos*—denoting a *stranger* or *foreign life*. An axenic organism, as here defined, is a species free from any life apart from that produced by its own protoplasm.

The writers are greatly indebted to Professor A. C. Johnson, Department of Classics, Princeton University, for suggesting and defining the term *axenic*.

5 Nuttall, George H. F., und Thierfelder, H., *Z. f. Physiol. Chem.*, 1895, **21**, 109.

6 Cohendy, Michel, *Ann. de l'Inst. Pasteur*, 1912, **26**, 106.

7 Wollman, Eugène, *Ann. de l'Inst. Pasteur*, 1913, **27**, 154.

8 Reyniers, J. A., in *Micrurgical and Germ-Free Methods*, Charles C. Thomas, Springfield, Ill., (to appear in 1942).

9 Trager, William, *Physiol. Rev.*, 1941, **21**, 1.

conditions. It is reasonable to expect that methods will eventually be found which will permit an axenic vertebrate to grow and reproduce. When one or more species of axenic higher animals have been reared to physiological maturity, both invertebrate and vertebrate material will be available for the study of important biological problems.

Present-day investigators who desire to rear axenic animals have several ends in mind (Glaser).[10] They may be interested in the exact nutritional requirements of an animal without having to consider the role played in the digestion of food by organisms normally inhabiting the digestive tract. They may wish to study the reaction of an animal to a single species of pathogenic agent. Or they may be interested in the mechanism of acquired immunity developing in a host without interference from antibodies resulting from life in a contaminated state.

Warm-blooded, comparatively large laboratory animals were used by all the various workers, with the exception of the experiments with tadpoles; the nutritional and reproductive difficulties should be more readily overcome in some small cold-blooded vertebrate because of the size and ease of handling. A technic was therefore devised for removing axenic young from a small viviparous fish and certain nutritional work was done. A description of the operation and the results obtained are presented.

Material and Methods. The platyfish *(Platypoecilus maculatus)* is a small tropical fish, 4 to 5 cm in length, which is found in abundance in Mexican waters. In this country it is obtained readily from commercial fish hatcheries. The female platyfish bears large numbers of living young (each about 1 cm long), usually about 20 every 4 weeks. Seasonal variations in reproduction have been observed and it appears that the largest number of young are produced in the spring and early summer.

The platyfish was found readily adaptable to certain types of laboratory experiments by Gordon,[11] in his genetical and tumor research, and Baker,[12] in his work on tuberculosis of platyfish. The small size of these fish, their adaptability, and the ease with which they could be kept in a laboratory environment led to the conclusion that they would be ideal subjects for work under sterile conditions. A method of operation was devised by which young could be removed from the mother without contamination.

The scales of the fish are covered by a continuous layer of epithelium that retains all bacteria on a relatively smooth surface (Fig. 2). By application of a germicidal agent to this surface, it was found that an area for operation could easily be made sterile. From studies of sagittal sections, it was known that the embryos are located posterior and dorsal to the intestinal tract (Fig. 3). Hence, by extracting the young through the ruptured walls of the ovary, contamination from the intestines could be avoided.

Pregnant females, determined by obvious distension of the abdomen, were immersed in alcohol for 3 min and then placed in tincture of iodine for 7 min. The iodine was removed by rinsing with alcohol and finally ether, which by evaporation gave a dry, sterile surface. The fish were fastened with a stapler to a board of balsa wood and the scales over the operating area were stripped off with forceps. An incision slightly posterior and dorsal to the anus (Fig. 1) was made in the abdominal wall and a sterile pipette (tip 3 mm in diameter) was introduced into the body cavity. Gentle suction with a rubber bulb caused rupture of the ovary, and drew a number of embryos into the pipette, from which they were transferred temporarily to a Petri dish containing sterile water. This procedure was repeated several times for each female, and usually 10 to 15 young were obtained. All of the young were not removed because it was feared that continued pressure might rupture the intestinal tract and cause contamination. The young

[10] Glaser, R. W., in *Micrurgical and Germ-Free Methods,* Charles C. Thomas, Springfield, Ill., (to appear in 1942).

[11] Gordon, Myron, *Am. J. Cancer,* 1937, **30**, 362.

[12] Baker, James A., *J. Inf. Dis.,* 1942, **70**, 248.

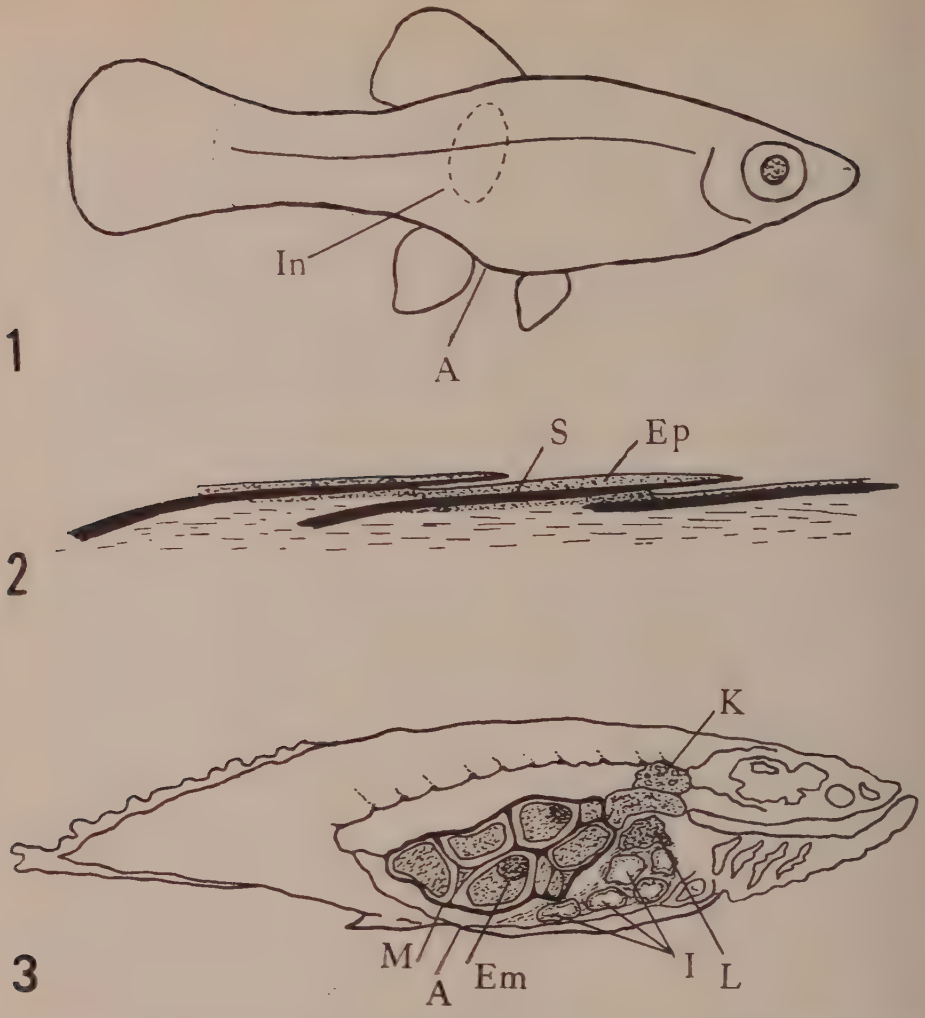

FIG. 1.

The drawings show: (1) Location of incision in body wall for removal of embryos; (2) Relation of epithelium to scales on body surface; (3) Location of embryos in pregnant fish with respect to other visceral organs as seen in sagittal section. A—anus; EM—embryo; EP—epithelium; I—loops of intestine; In—location of incision; K—kidney; L—liver; M—ovarian wall; S—scale.

fish were distributed singly or in pairs into cotton-plugged bottles containing ½ to 2 liters of autoclaved tap or pond water. Small amounts of sterile food were given at semi-monthly or monthly intervals and the fish were observed to eat in a normal manner.

Nutrition. It can be seen from the table that fish fed agar particles lived for 2 to 3 weeks without apparent growth and died when the contents of the yolk sac were depleted. The decline was gradual with progressive emaciation and weakness signaling death. Those fed an autoclaved, commercially prepared fish food, known to be adequate under usual aquarium conditions, lived for a period longer than 2 months and showed some increase in size. Growth was rapid during the first month but it gradually ceased. Thereafter the fish appeared to be in a static condition for 2-3 weeks and then

declined as if unfed. Additional growth and longer survival were obtained in fish given nematodes *(Neoaplectana glaseri)* that had been reared free from microörganisms.[13] A diet of autoclaved fish food plus nematodes was not markedly superior to that of nematodes alone. Nematodes supplemented with heat-killed algæ or autoclaved housefly larvæ proved to be the best diets in that life was maintained longer than 4 months, but they were not completely satisfactory. Pond water showed no advantage over tap water, and a change of water did not promote better growth. Probably certain nutritional requirements were not supplied.

TABLE I.

Effect of Various Foods on Size and Survival of Axenic Platyfish.

Food	Effect on fish: Size, cm	Effect on fish: Survival, days
Agar particles	1	14- 21
Commercial fish food	2	60- 90
Commercial fish food + nematodes	2-2.5	75-120
Nematodes	2-2.5	75-109
Nematodes + heat-killed algæ	2-2.5	93-127
Nematodes + autoclaved housefly larvæ	2-2.5	113-127

[13] Glaser, R. W., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 512.

Bacteriological Examination. Whole fish, as well as samples of the water in which they had lived, were tested for contaminating microörganisms under aerobic and anaerobic conditions by culture in infusion broth and sealed piece-meat media incubated at both room temperature and 37°C. Practically all of the fish had remained free of microorganisms. Molds occasionally developed in some containers, and, more rarely, bacterial contamination occurred. Usually it was possible to determine contamination merely by inspection of the aquariums, which showed molds growing or a marked turbidity when bacteria were present.

Summary. An operative procedure on pregnant platyfish, a small viviparous Mexican fish, was devised that regularly yielded young free from contamination. These axenic fish were maintained under conditions free from microörganisms for as long as 4 months and during this period grew appreciably. Under usual aquarium conditions these fish would have reproduced at this age. Several diets were tested for their effect on maintenance and growth and some of them were definitely superior to others—an indication that nutritional factors may be the principal cause of the lack of continued growth and reproduction.

13855

Mechanism of Vitamin B_1 Destruction by a Factor in Raw Smelt.

H. F. DEUTSCH AND G. L. OTT. (Introduced by H. P. Rusch.)

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The destruction of vitamin B_1 by certain species of fish when incorporated into a diet in the raw form has aroused considerable interest. The work of Green *et al.*[1-4] on carp has treated the nutritional aspects of this problem in some detail. Recent work by Woolley[5] on the causative agent of this destruction in fresh carp indicated either an enzymatic degradation or a tying up of the thiamine molecule in such a manner that it is not available to the living organism. Spitzer *et al.*[6] likewise suggest that the destruction may be due to an enzyme.

We have investigated the vitamin B_1 de-

[1] Green, R. G., Carlson, W. E., and Evans, C. A., *J. Nutrition*, 1941, **21**, 243.

[2] *Ibid.*, 1942, **23**, 165.

[3] Green, R. G., and Evans, C. A., *Science*, 1940, **92**, 154.

[4] Green, R. G., Evans, C. A., Carlson, W. E., and Swale, F. S., *J. Am. Vet. Med. Assn.*, 1942, **100**, 394.

[5] Woolley, D. W., *J. Biol. Chem.*, 1941, **141**, 997.

structive factor in raw fresh water smelt by studies on the correlation of chemical and animal assays for this factor, on the quantitative action of raw smelt on thiamine, on the ability to obtain the factor in an alcoholic extract, on its lability to heat and drying, on its action on the thiamine of living yeast cells, and by attempts to release thiamine from any complexes by acid hydrolysis.

Procedure. A dry non-viable yeast* and a wet viable yeast† were used as the source of vitamin B_1. The dry non-viable yeast was mixed with either raw smelt, heated smelt (100°C for 10-30 min), alcoholic (5%) extracts of raw smelt, the residues of the alcoholic extracts, and air-dried smelt restored to its original moisture content. Non-viable yeast - raw smelt mixtures of varying ratios were prepared to determine the quantitative vitamin B_1 destructive effect of smelt. Moist, viable baker's yeast was suspended in saline and a portion of the suspension was killed by heating at 95°C for 15 min. Both the living and killed suspensions were thoroughly mixed with portions of raw smelt to give a vitamin B_1 - fish ratio analogous to the lowest level of the non-viable yeast-smelt mixtures. These mixtures were air-dried at room temperature under forced ventilation. The moist period of contact of smelt and yeast was approximately 12 hr.

After thorough drying, the smelt-yeast preparations were added to a diet in which the thiamine had been destroyed by prolonged autoclaving. These dry fish-yeast mixtures were incorporated in amounts sufficient to provide, on the basis of the yeast content, an amount of vitamin B_1 greatly in excess of that reported by Elvehjem and Arnold[7] as being necessary for normal chick growth. Day-old chicks were placed on these diets. Two groups of chicks on a diet containing the raw smelt - non-viable yeast mixtures received daily intraperitoneal injections of 3 and 6 γ of thiamine hydrochloride respectively.

These diets were then analyzed for thiamine by the thiochrome method. Samples of the raw smelt-yeast mixtures were heated in 0.1 N H_2SO_4 at 100°C for periods ranging from 10-60 min and then assayed for thiamine by the thiochrome method.

Results. Chemical analyses of the diets revealed that only those containing cooked smelt or raw smelt-viable yeast mixtures retained the approximate amount of added vitamin B_1. Some of the thiamine in the viable yeast-fish mixtures was destroyed since the recovery is less than can be explained as due to variations encountered in the thiochrome procedure.

The destruction of vitamin B_1 was complete in all other fish-yeast mixtures except in the preparation having 43.7 γ of vitamin B_1 per g of raw smelt, in which case a small amount of thiamine was recovered in the diet. The results of the chick assays were in complete agreement with the chemical analyses. Typical polyneuritic symptoms developed in chicks on the ninth day after being placed on the diets shown to be free of vitamin B_1 by chemical analysis. Those chicks on the diet containing a trace of vitamin B_1 (3.4 γ per 100 g of ration) survived approximately one day longer. As little as 3 γ of thiamine hydrochloride injected daily protected the chicks on the vitamin B_1 free diets from polyneuritis although this represented a bare maintenance level as indicated by their growth gains. The results are shown in the table.

Treatment of dried raw smelt - non-viable yeast mixtures with 0.1 N H_2SO_4 for as long as 60 min failed to free any thiamine which could be detected as thiochrome.

Discussion. By chemical analysis of raw fish - non-viable yeast mixtures which have been in contact for a sufficient period of time, it is possible to determine rapidly whether certain species of fish will destroy vitamin B_1. In the experiments herein de-

[6] Spitzer, E. H., Coombes, A. I., Elvehjem, C. A., and Wisnicky, W. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 376.

* A dry, killed brewer's yeast assaying 140 γ thiamine per g of yeast.

† A wet, living baker's yeast assaying approximately 205 γ thiamine per g. Furnished through courtesy of Red Star Yeast Co.

[7] Arnold, A., and Elvehjem, C. A., *J. Nutrition*, 1938, **15**, 403.

TABLE I.
Correlation of Chick and Thiochrome Assay for Vitamin B_1 Destruction by Smelt.

Smelt treatment	γ B_1 (as yeast)/g raw smelt (or equivalent)	Calc. γ B_1 (as yeast by addition of smelt-yeast mixt.)/100 g ration	γ B_1/100 g found by chem. assay*	Polyneuritis in chicks‡
Cooked	8.7 (Brewer's)	238	219	—
Raw	8.7 "	238	0	+†
"	21.9 "	458	0	+
"	43.7 "	661	3.4	+
"	9.6 (Baker's)	357	318	—
"	9.6 (Killed baker's)	357	0	+
Air dried	8.7 (Brewer's)	238	222	—
" " + water	8.7 "	238	217	—
5% alc. extr.	8.7 "	238	0	+
Residue from alc. extr.	8.7 "	238	0	+

*Assayed by thiochrome procedure.

†Two groups of 5 chicks on this diet received daily intraperitoneal injections of 3 and 6 of thiamine hydrochloride daily and failed to show any polyneuritic symptoms in 15 days.

‡Eight chicks per group except injection group. Mortality indicated by + was 100%, — no mortality.

scribed the period of contact was determined by the time necessary to dry the fish-yeast mixtures in thin layers under forced ventilation at room temperature. Recent experiments have shown that this period was much longer than necessary to effect marked disappearance of thiamine. No attempt was made, however, to measure the rate of destruction. The greatest amount of destruction that occurred in the mixtures containing 43.7 γ B_1 per g of raw fish does not necessarily represent the highest level of destruction possible. Undoubtedly by using higher levels of vitamin B_1 and longer periods of contact, a greater destruction per unit weight of fish could be obtained.

The vitamin B_1 destruction by certain species of raw fish is interesting in view of the tying up of another vitamin, biotin, by a protein factor in raw egg white. Unlike biotin, it does not appear that the thiamine inactivation is due to the formation of an analogous complex by a protein factor in the raw fish, since acid hydrolysis does not release any thiamine from the smelt-thiamine mixtures. The destructive factor appears to destroy easily both the free form of thiamine and its pyrophosphate. Crystalline thiamine hydrochloride and the pyrophosphate ester which makes up a large portion of the vitamin B_1 of dried, killed brewer's yeast, is completely destroyed within the stated limits by a factor in the raw smelt.

Woolley[5] states that the mechanism of vitamin B_1 inactivation by carp is not due to a splitting of the thiamine molecule into its pyrimidine and thiazole portions. It is possible, however, that the destructive factor might degrade either the pyrimidine or thiazole portion after a preliminary hydrolysis of the molecule. The failure of thiamine to form thiochrome after treatment with raw smelt and the absence of a hydrolysis labile thiamine-protein complex strongly suggests that the destruction is in the nature of an enzymatic degradation.

The failure of raw smelt to destroy the thiamine in living yeast is due very likely to the inability of the destructive factor to penetrate the living yeast cell. It appears that a portion of the vitamin B_1 in living yeast was destroyed (see table) but this is probably due to the presence of a certain amount of non-viable yeast in the baker's yeast preparation used. Destruction of the living yeast by heating rendered its B_1 content labile. Woolley and Longsworth[8] have shown that the antibiotin factor was ineffective in hindering initiated yeast growth. The antithiamine factor appears to be similar in its inability to penetrate the living cell.

[8] Woolley, D. W., and Longsworth, L. G., *J. Biol. Chem.*, 1942, **142**, 285.

A portion of the active destructive agent in raw smelt can be extracted with 5% alcohol. No attempts were made at a quantitative extraction. The residues after alcohol extraction showed high activity. Spitzer *et al.*,[6] on the basis of the chick assay, report that aqueous extracts of carp are inactive while ether extracts showed partial activity. The carp residue after water and ether extraction showed partial activity. Woolley[5] reports that aqueous extracts of carp had 25% of the activity of whole carp suspensions and that 10% NaCl extracts of carp were highly active.

When finely pulverized smelt are thoroughly dried at room temperature, the B_1 destructive factor is lost and subsequent replacement of the original moisture content will not restore this factor. Heating smelt at 100°C for 5 min also causes a complete destruction of the active factor.

The use of certain species of raw fish in the preparation of vitamin B_1 free diets to be used for experimental purposes appears to be worthy of further investigation. Present methods of preparing B_1 free rations by autoclaving for considerable periods of time undoubtedly cause the destruction or alteration of other dietary factors. The use of a fish or a fish extract would circumvent these undesirable effects.

Summary. A viable yeast can protect its thiamine from destruction by a factor present in raw smelt. The antithiamine factor behaves similarly to the antibiotin factor in its inability to penetrate the living cell. The inactivation of thiamine does not appear to be due to the formation of thiamine complexes labile to hydrolysis or to the splitting of the B_1 molecule but rather to an enzymatic degradation of the thiamine molecule. The destructive factor in raw smelt is labile to air drying at room temperatures, heating at 100°C and is extractable in 5% alcohol. Certain raw fish or extracts of these fish may prove valuable in the preparation of vitamin B_1 free diets without the necessity of resorting to prolonged autoclaving.

13856

Estimation of Reduced Degradation Products in Experimental Trinitrotoluene Poisoning.

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Dale[1] presented tentative evidence that administered TNT (trinitrotoluene) in rabbits is partly reduced to dinitro-azoxytoluene and partly to amino compounds. The work of Voegtlin, Hooper and Johnson[2] indicated that in man the portion of the urinary material giving the Webster[3-5] test was the hydroxylamino derivative. It would seem all of these should, under proper conditions, be diazotizable and if coupled with a suitable color reagent, lend themselves to colorimetric estimation. Elvove[6] made a step in this direction, but his method involved reduction of residual nitro groups to nitrite, and application of the Griess reagent. The widely used Webster test suffers from several severe handicaps: (a) the test has

1 Dale, H. H., Medical Research Council of Great Britain, Special Rept. Series No. 58, 1921, 52.

2 Voegtlin, C., Hooper, C. W., and Johnson, J. M., Hygienic Laboratory Bull. No. 126, 1920.

3 Webster, T. A., *Lancet*, 1916, **191**, 1029.

4 Tutin, F., *Lancet*, 1918, **195**, 554.

5 Ingham, J., *Lancet*, 1941, **241**, 554.

6 Elvove, E., *J. Ind. Eng. Chem.*, 1919, **11**, 860.

low sensitivity, hence, is of little value for small quantities, (b) it is negative with the urine of some carnivora such as the cat, (c) it apparently becomes negative in the urine of the human subject with the onset of grave symptoms, (d) it does not by color alone distinguish unchanged TNT from the conjugated degradation products, (e) it is not quantitative. The procedure presented in this paper indicates about the same percentage excretion of reduced products by various animals including those carnivora which give a negative Webster test, it furnishes a method for detecting the residual chromogen in urine after the Webster chromogenic material is extracted, and is sensitive enough to permit tissue and blood analyses.

The test consists essentially in the diazotization of the reduced products formed by the body and subsequent coupling with the color reagent of Bratton and Marshall.[7] Obviously other compounds such as the sulfa drugs or nitro compounds yielding diazotizable groups might interfere. TNT as such does not.

A suitable aliquot of the 24-hr urine (0.05 to 0.3 cc for animals getting 50 mg/kg daily subcutaneously or 0.3% in the diet) is measured into a graduated cylinder, water added to 7.5 cc and 2.5 cc 4 N HCl added. The cylinders are immersed in ice water for 10 min, 1 cc of sodium nitrite (0.1%) added, mixed, allowed to stand 2 min, 1 cc ammonium sulfamate (0.5%) added, mixed, allowed to stand 2 min and 1 cc N-(1-naphthyl) ethylene diamine dihydrochloride (0.1%) added. At the same time standards containing 10, 20, 30, and 40 μg 6-nitro 2, 4-diamino-toluene are prepared and diazotized in the same way. For samples where the concentration is low, color comparison is better if the standard contains an equivalent amount of urine from a control animal. Comparison is made after 5 min. Tissues may be extracted with trichloracetic acid of suitable concentration and the filtrates diazotized. For certain purposes it is best to prepare acetone filtrates (1 part tissue, 3 parts absolute acetone) of the tissues since TNT and some derivatives are very sparingly soluble in acid solution. After evaporation of the acetone at room temperature, the unchanged TNT may be extracted from an aqueous 1% bicarbonate suspension with ether and estimated by the method of Pinto and Fahy[8] or that of Kay.[9] Rabbits apparently excrete a portion of the administered TNT as a conjugated dinitro-hydroxylamino-toluene; hence, color comparison is not as satisfactory as for cat's urine. A 20-80 mixture of the amino and hydroxylamino compounds might be used as standard. For the ether extract prepared by the Webster method 2,6-dinitro-4-hydroxylamino-toluene has been found to more nearly approximate the color. For the dog, 2,4,6-triaminotoluene appears to be the most suitable standard.

With this procedure a rabbit gave diazotizable material in the urine for 3 weeks, and in the liver at necropsy, following a single tolerated dose of 0.4 g per kg. Representative of findings with the method are those for rabbit number 7 dying 48 hr after a single toxic dose, as shown in Table I.

TABLE I.

Distribution of the Reduced Material in the Rabbit 48 Hours Following a Single Toxic Dose of TNT of 500 mg/kg Subcutaneously

Organ or fluid	Mg/100 g or cc as the Diamino Cpd
Blood	6.5
Liver	2.0
Kidney*	5.0
Muscle	1.0
Bile	2.0
Fat	1.0
Lung	3.0
Spleen	3.0
Bone Marrow	2.5
Testicles	0.5
Aqueous humor	0.5
Urine*	330.0

*Webster test positive on kidney and urine.

Fat was the only tissue which on extraction with acetone, evaporation of the acetone, suspension of the residue in 1% $NaHCO_3$ and extraction with ether gave a positive test for unchanged TNT with Webster's reagent.

[7] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

[8] Pinto, S. S., and Fahy, J. P., *J. Ind. Hyg. and Toxicol.*, 1942, **24**, 24.

[9] Kay, K., *Canadian J. Research*, 1941, **19**, 86 (Section B).

Summary. A method has been described for tracing reduced degradation products of trinitrotoluene in experimental animals. The role of these products in trinitrotoluene poisoning remains to be determined.

13857

Transmission of the Murine Strain of Poliomyelitis to the Syrian Hamster.

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The Lansing strain of poliomyelitis has been successfully passed to the cotton rat and mouse by Armstrong.[1,2] Since certain studies, such as serological investigations, can be performed on these animals with difficulty, it was considered of interest to try to pass the mouse strain to a larger animal. After the mouse-adapted strain had been successfully passaged through 16 Syrian hamster passages, we learned of Armstrong's work where he reports passing the virus through a few hamsters.[3] Since no description of the disease is given it is considered of interest to report our results here.

The 211th Armstrong mouse brain passage was employed to initiate these studies. 0.25 cc of a 1 to 10 mouse brain suspension was inoculated intracerebrally into young hamsters. After 4 days one hamster became paralyzed in both hind legs. The brain and cord were then passed serially to other hamsters and mice. Table I indicates the results obtained.

It is seen that the virus has gone through 16 successive hamster brain passages. Of 75 inoculated hamsters, 46 or 61.3% developed paralysis and died after an incubation period varying from 2 to 15 days with an average period of incubation of 5⅕ days. Only 7 of the paralyzed animals survived. The 1st, 2nd, 4th, 5th, 9th, and 12th hamster brain passages were inoculated into mice and in all instances mice from each series developed typical mouse poliomyelitis after an average incubation period of 7⅓ days.

The disease in the hamster runs the following course. After 24 hr the animal may appear lethargic and irritable while recovering from the effects of the inoculation, but usually they are bright eyed and inquisitively active, quite like a normal animal. The onset of the disease is variable. The first symptoms are usually irritability and malaise followed by a swelling of the eyelids, so that they are nearly closed. Paralysis usually occurs 1 or 2 days after the appearance of the first symptoms. The extent of paralysis is quite variable, from slight irregularities in gait, paralysis of a few toes on one foot to complete paralysis of the legs. Fourteen died of respiratory paralysis while the others died after paralysis of the fore or hind legs. During advanced stages of paralysis, the animal appears moribund, breathes heavily and irregularly and moves only slightly when disturbed. Death, through respiratory paralysis, may occur within 1 or 2 days following paralysis of the legs. In those animals that recovered, there was a permanent paralysis, followed by atrophy and contractures.

Grossly the brain and cord show no abnormalities. Microscopically, there is a diffuse perivascular cuffing of the intrinsic cerebral vessels, occasional foci of diffuse leucocytic infiltration are seen with greatest frequency and greatest intensity in the cerebral peduncles. There is in addition, a focal,

[1] Armstrong, C., *Public Health Report*, 1939, **54**, 1719.

[2] Armstrong, C., *Public Health Report*, 1939, **54**, 2302.

[3] Armstrong, C., and Packchanian, unpublished data, referred to in Vanderbilt Lectures, April, 1941. Armstrong informed us that 2 or 3 passages were made.

TABLE I.
Hamster Passages of Polio Virus.

Hamster passages						Mouse passages (from corresponding hamster passage)		
		Animals paralyzed (or dead)					Animals paralyzed (or dead)	
Passage No.	No. of animals inoculated	No.	%	Days after inoculation when paralysis occurred	No. of animals recovering from paralysis	No. of animals inoculated	No.	%
1	3	1	33.3	4		6	3	50
2	3	1	33.3	7		4	4	100
3	7	4	57.1	2-4-4-5				
4	6	1	16.6	5		5	4	80
5	5	3	60	3-4-5		5	5	100
6	5	4	80	2-2-4-15	1			
7	3	2	66.6	2-2				
8	4	3	75	3-4-14	1			
9	4	1	25	3		6	5	83.3
10	2	1	50	3				
11	8	7	87.5	3-3-3-4-4-5-5	2			
12	6	6	100	2-2-2-6-6-10	1	7	7	100
13	5	4	80	3-3-3-3	2			
14	6	5	83.3	9-11-11-11-12				
15	3	1	33.3	4				
16	5	2	40	3-15				
Totals	75	46	61.3%	5.2 days	7			

predominantly round cell meningitis. In the sections of spinal cord several small contracted ganglion cells in the anterior horns, suggestive of early necrosis are seen. In addition there is associated polymorphonuclear leucocytic infiltration in the anterior horn segments of several levels. Several vessels within the white matter show marked perivascular cuffing.

Neutralization tests were performed employing hamster brain virus and normal human serum as well as human convalescent poliomyelitis serum. The test animal was the mouse. The positive human convalescent serum neutralized the hamster virus while the normal human serum did not. Likewise, hamster convalescent serum neutralized the hamster virus as well as the mouse virus.

The 5th hamster brain passage was inoculated intracerebrally into 2 monkeys, each receiving 0.5 cc of a 1:10 suspension of brain material. The monkeys showed no signs of illness. After 1 month these monkeys and 1 control monkey were tested with a potent monkey poliomyelitis cord virus, all receiving 0.5 cc of a 1:10 cord suspension, by intracerebral inoculation. The monkeys, previously inoculated with the hamster virus, proved to be immune, while the control monkey developed paralysis and died on the 9th day. The 16th hamster brain passage was inoculated into 2 monkeys, each receiving 0.5 cc of a 1:10 brain suspension, inoculated intracerebrally. In both instances the temperature rose on the 6th day but there was no paralysis. One monkey was sacrificed on the 9th day and a 10% suspension of cord was inoculated intracerebrally into another monkey. This monkey did not develop disease nor was it immune to a subsequent inoculation of potent monkey poliomyelitis virus. The surviving monkey, from the 16th hamster brain inoculation, was tested for immunity after 28 days. This monkey, as well as a control monkey, received 0.5 cc of a 10% suspension of potent monkey cord virus, intracerebrally. The control monkey died of poliomyelitis after 7 days, while the other monkey proved immune.

Conclusions. The Armstrong mouse-adapted poliomyelitis strain was successfully passed serially through 16 Syrian hamster brain passages. The disease in hamsters is usually characterized by paralysis of one or

two limbs and followed by death. A small number of paralyzed hamsters survived, but developed atrophy and contractures of the affected muscles. The pathology in the infected hamsters is typical of poliomyelitis. The hamster strain is infectious for mice. Human convalescent poliomyelitis serum neutralized the hamster strain. Hamster convalescent serum neutralized the hamster strain as well as the mouse strain. The hamster strain inoculated intracerebrally into 4 monkeys produced no paralysis. Three monkeys, subsequently tested with virulent monkey cord virus, proved to be immune.

The authors wish to thank Dr. C. Armstrong for the mouse-adapted strain as well as Colonel Ash, Army Medical Museum, for examining the sections.

13858

Antimicrobial Action of Pyocyanine, Hemipyocyanine, Pyocyanase, and Tyrothricin.

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The isolation of substances from various bacteria, fungi and actinomycetes which in extremely small amounts inhibit or destroy many types of pathogenic bacteria, has aroused considerable interest in recent years. These include such substances as pyocyanase,[1] pyocyanine,[1] hemipyocyanine,[1] tyrothricin[2] (and its components, gramicidin and tyrocidine), actinomycin,[3] streptothricin,[4] gliotoxin,[5] and penicillin.[6]

In view of the present marked interest in antibiotic substances of microbial origin and because of the availability of pyocyanase, pyocyanine and a number of synthetic pyocyanine salts as well as hemipyocyanine, it appeared to be of some interest to compare the antimicrobial activities of these substances with each other and to some extent with tyrothricin.

Hemipyocyanine* (α-hydroxyphenazine), pyocyanine hydrochloride and pyocyanine methosulfate (monomethyl sulfuric acid salt of pyocyanine) were prepared by synthetic methods.[7] Pyocyanine was also isolated as the phenazonium compound and as the hydrochloride and as the perchlorate from cultures of *Ps. aeruginosa.* Pyocyanase which is a crude ether-soluble fatty material composed of a mixture of substances, probably including some pyocyanine, was isolated from the same cultures. Tyrothricin was prepared according to Dubos and Cattaneo.[8]

Bacteriostatic activity was determined by thoroughly mixing varying amounts of each substance with 10 ml of melted brain-heart infusion agar in Petri dishes. After the agar had solidified, the surface was streaked with a loopful of an 18-24 hr broth culture of each test organism. The presence or absence of growth was noted after the plates had been incubated at 37°C for 24 hr. The inhibitory values given in Table I are probably somewhat low since, in most cases, the next dilution used at which growth occurred was twice the inhibitory dilution. It is evi-

1 Kramer, H., *Z. Immunitäts.*, 1935, **84**, 505.

2 Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

3 Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1941, **42**, 231.

4 Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1942, **43**, 9.

5 Dutcher, J. D., *J. Bact.*, 1941, **42**, 815.

6 Chain, E., *et al., Lancet*, 1940, **239**, 226.

* We are indebted to Dr. M. Tishler of Merck & Co., Inc., for supplies of hemipyocyanine and pyocyanine perchlorate.

7 Wrede, F., and Strack, E., *Ber.*, 1929, **62**, 2053; *Z. physiol. Chem.*, 1928, **177**; 1929, **181**, 58; also Hilleman, H., *Ber.*, 1938, **71**, 34. See also Einhorn, A., *et al., Ber.*, 1904, **37**, 106.

8 Dubos, R. J., and Cattaneo, C. J., *J. Exp. Med.*, 1939, **70**, 249.

TABLE I.
Comparative Bacteriostatic Activity of Pyocyanines, Hemipyocyanine, Pyocyanase and Tyrothricin.

Organisms	Natural pyocyanine (perchlorate)	Synthetic pyocyanine (hydrochloride)	Synthetic pyocyanine (methosulfate)	Synthetic hemi-pyocyanine	Pyocyanase	Tyrothricin
	Dilution at which growth is inhibited × 1000					
S. aureus	40	40	40	10	10	40
S. albus	20	20	20	5	5	40
S. viridans	20	20	20	5	5	1000
S. lactis	20	20	20	20	5	—
S. hemolyticus	100	100	100	10	10	1000
N. catarrhalis	100	100	100	20	10	1.6
S. aertrycke	10	10	10	<5	<5	—
S. paratyphi	20	20	20	5	10	1.2
S. schottmuelleri	20	20	20	<5	5	—
E. coli	10	10	10	5	5	1.2
E. typhosa	20	20	20	5	5	1.2

dent that a dilution of 1:10,000 or greater of natural or synthetic pyocyanine inhibited the growth of all bacteria tested; the Gram negative rods were about as sensitive as the Gram positive cocci. *S. hemolyticus* and *N. catarrhalis* were highly susceptible to the action of pyocyanine as shown by the complete inhibition of growth at a dilution of 1:100,000. Synthetic pyocyanine and the pyocyanine isolated from natural sources were identical in activity, as would be expected. Pyocyanine methosulfate, pyocyanine hydrochloride and perchlorate had the same activity on a molar basis, which indicates that the bacteriostatic properties of these compounds are due entirely to the pyocyanine moiety.

Hemipyocyanine has considerably less inhibitory action than pyocyanine. Most of the bacteria were inhibited at a dilution of 1:5,000 or less. The difference between the activities of pyocyanine and hemipyocyanine is brought out sharply in the case of *N. catarrhalis* and *S. hemolyticus*. While pyocyanine inhibited these organisms at a dilution of 1:100,000, it was necessary to use 5 to 10 times as much of hemipyocyanine to stop their growth.

The bacteriostatic potency of pyocyanase closely paralleled that of hemipyocyanine and was, therefore, considerably less than that of pyocyanine.

The specificity of the bacteriostatic properties of tyrothricin is clearly indicated. Although *S. viridans* and *S. hemolyticus* were inhibited at a 1:1,000,000 dilution and the staphylococci at 1:40,000, the Gram negative rods, *E. coli, E. typhosa* and *S. paratyphi* failed to grow only at a dilution of 1:1200 and *N. catarrhalis* at 1:1600.

All of these substances have much greater bacteriostatic potency than the sulfonamides. Sulfanilamide, sulfapyridine and sulfathiazole when tested in the same manner failed to inhibit the growth of any of the bacteria at a dilution of 1:1000.

An interesting reversal of order of activity is found in Table II which contains results obtained with a number of different yeasts. Pyocyanine perchlorate and pyocyanine hydrochloride were unable to inhibit the growth of the yeasts at a dilution of 1:5000; pyocyanine methosulfate was somewhat more active since most of the yeasts were inhibited at a dilution of 1:10,000. Hemipyocyanine which had been relatively inactive toward bacteria was the most active inhibitory compound towards the yeasts. A dilution of

TABLE II.
Inhibition of Yeasts by Pyocyanines, Hemipyocyanine and Pyocyanase.

Yeasts	Natural pyocyanine perchlorate	Synthetic pyocyanine hydrochloride	Synthetic pyocyanine methosulfate	Synthetic hemi-pyocyanine	Pyocyanase
	Dilution at which growth is inhibited × 1000				
Torulopsis kefir	<5	<5	40	100	40
Zygosaccharomyces Marxianus	<5	<5	10	100	5
Saccharomyces fragilis	<5	<5	10	40	5
Schwanniomyces occidentalis	<5	<5	10	40	40
Saccharomyces cerevisiæ	<5	<5	10	100	40
Saccharomyces ellipsoideus	<5	<5	10	100	40

1:100,000 of hemipyocyanine was bacteriostatic for most of the yeasts. These results emphasize strongly the specificity of action of antibiotic substances of microbial origin.

Pyocyanase inhibited the growth of the yeasts at higher dilutions than the pyocyanines but was not as active as hemipyocyanine.

Studies on the activity of antibiotic agents of microbial origin have been almost entirely confined to their action on bacteria. We, therefore, have very little information concerning their effects on fungi. Many of these agents in their present form cannot be used intravenously because of hemolytic properties, toxicity or because they are ineffective by this route. Their therapeutic use is thus limited to surface or localized infections which are directly accessible. Fungus infections in man are predominantly of this type and may represent an important field of application for microbial therapeutic substances.

The fungistatic properties of pyocyanine (perchlorate), hemipyocyanine and tyrothricin towards the pathogenic fungi: *Achorion schoenleinii, Microsporium gypseum, Trichophyton gypseum* and *Candida albicans,* were determined.† Sterile water dilutions of the microbial agents were added to 5 cc of sterile Sabouraud's broth contained in 25 cc Erlenmeyer flasks. The fungi were cultured on Sabouraud's agar for 1-2 weeks. Suspensions of the fungi were made by scraping off a small amount of growth and suspending it in sterile water. Each flask of medium was inoculated with 0.1 cc of suspension. All cultures were incubated in 28°C for 7 days after which the presence or absence of growth was recorded. The results are given in Table III. It is evident that both hemipyocyanine and tyrothricin, in relatively high dilutions, completely inhibited the growth of the pathogenic fungi while pyocyanine was considerably less effective. Tyrothricin completely inhibited all of the pathogenic fungi at dilutions of 1:5000 to 1:20,000; pyocyanine from 1:2000 to 1:5000; and hemipyocyanine from 1:20,000 to 1:60,000. *Achorion schoenleinii* was the most sensitive of the fungi to the agents while *Candida albicans* was the most resistant fungus. No attempt was made to equalize the amount of inoculum used and this may be a factor in the difference in resistivity of the fungi. It is interesting to note that hemipyocyanine which was most active against yeasts was also the most active compound against fungi.

Although it is difficult to compare data of this type with that of other investigators because of differences in medium, strain of

† We are indebted to Dr. N. F. Conant, Medical School, Duke University, for strains of *Monilia albicans, Achorion schoenleinii,* and *Trichophyton gypseum,* and to Dr. R. Benham, Dept. of Bacteriology, Columbia College of Physicians and Surgeons, for the strain of *Microsporium.*

TABLE III.
Fungistatic Properties of Tyrothricin, Pyocyanine and Hemipyocyanine.

Dilution	*Achorion schoenleinii*	*Microsporium gypseum*	*Trichophyton gypseum*	*Candida albicans*
		Tyrothricin.		
1:2000	—	—	—	—
1:5000	—	—	—	—
1:10,000	—	—	—	++++
1:20,000	—	+	+	++++
1:40,000	+	++	+	++++
1:60,000	+	++	+	++++
1:80,000	++++	+++	+++	++++
1:100,000	++++	+++	+++	++++
Control	++++	++++	++++	++++
		Pyocyanine.*		
1:2000	—	—	—	++
1:5000	—	+	+	+++
1:10,000	++	++	++	+++
1:20,000	++	++	++	++++
1:40,000	++	+++	++++	++++
1:60,000	++++	+++	++++	++++
1:80 000	++++	++++	++++	++++
1:100,000	++++	++++	++++	++++
Control	++++	++++	++++	++++
		Hemipyocyanine.		
1:2000	—	—	—	—
1:5000	—	—	—	—
1:10,000	—	—	—	—
1:20,000	—	—	—	—
1:40,000	—	—	—	++
1:60,000	—	++	+	++++
1:80,000	++	++++	+	++++
1:100,000	+++	++++	++	++++
Control	++++	++++	++++	++++

*Pyocyanine perchlorate.
— No growth.
+ to ++++ Increasing amount of growth.

fungus, size of inoculum, etc., which greatly influence the results obtained, nevertheless it appears that the *in vitro* fungistatic potency of tyrothricin, and to a considerable extent of pyocyanine, is equal to or superior to that of such common disinfectants as mercuric chloride, phenol, cresol, salicylic acid, mercurochrome, etc. These inhibit the growth of various pathogenic fungi under similar conditions at dilutions generally less than 1:10,000.[9-10] The activity of hemipyocyanine is definitely superior to that of the commonly used disinfectants with the possible exceptions of crystal and gentian violet. All three antibiotic agents appear to be more effective than sulfanilamide, sulfapyridine, sulfathiazole and their sodium salts.[11]

[9] Schamberg, J. F., and Kolmer, J. A., *Arch. Dermat. Syphilol.*, 1922, **6**, 746.

[10] Gomez-Vega, P., *Ibid.*, 1935, **32**, 49.

[11] Lewis, G. M., and Hopper, M. E., *Ibid.*, 1941, **44**, 1101.

Discussion. There has been some tendency to describe antagonistic substances derived from microörganisms as being sharply differential in their effectiveness towards Gram positive and Gram negative bacteria. Such a demarcation is a useful one in many instances. Actually, however, as our results indicate and as is evident in the literature on penicillin, streptothricin and actinomycin, we are dealing, in the case of each substance, with a "spectrum" of activity ranging from low to high values depending upon the particular bacterium or type of microörganism involved. The greatest contrast in sensitivity, based on Gram straining properties was obtained with tyrothricin since, without exception, the Gram positive bacteria were about 40 to 1000 times as susceptible to this agent as members of the Gram negative group. However, even here the differentiation is not an absolute one since the Gram negative bacteria were also

inhibited in the presence of a sufficiently high concentration of tyrothricin. On the contrary, there was little or no correlation between Gram staining ability and the bacteriostatic potency of the pyocyanines, hemipyocyanine and pyocyanase.

The inhibitory action of the antibiotic substances studied is not confined to bacteria but extends to other groups of microorganisms such as yeasts and fungi. High inhibitory activity towards one type of microorganism does not necessarily signify similar potency towards other types: hemipyocyanine was much more inhibitory for yeasts and fungi than for bacteria.

Summary. The inhibition of growth of bacteria, yeasts and pathogenic fungi by natural and synthetic pyocyanine, synthetic hemipyocyanine, and natural pyocyanase and tyrothricin has been investigated. Natural and synthetic pyocyanine have equal activities which surpass those of pyocyanase. Tyrothricin is particularly inhibitory for Gram positive bacteria, especially streptococci. The considerable fungistatic potency of tyrothricin and hemipyocyanine suggests that these substances may be of value in the treatment of fungus infections.

13859

Protein Content of Rabbits' Aqueous Humor Following Intravenous Injection of *E. coli* toxin.*

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It has been shown[1,2] that culture filtrates of Gram-negative microörganisms capable of producing the Shwartzman phenomenon[3] (such as meningococcus, coli, typhoid, Shiga, proteus, and choleræ toxins) produce in rabbits a primary reaction characterized by iridoconjunctival hyperemia, and, upon removal, coagulation of the aqueous humor. This reaction appears several minutes after intravenous administration of toxin and reaches a maximum intensity in a few hours. The ocular symptoms are bilateral, usually of equal intensity, and disappear in the ensuing 24 hr.

In view of the possible bearing of this reaction on the problem of uveitis and other intraocular conditions, such as epidemic dropsy, the influence of intravenous injection of *E. coli* toxin on the protein content of the aqueous humor of rabbits has been investigated.

Methods and Material. A strain of *E. coli communior,* freshly isolated from a case of purulent cholecystitis, was inoculated in nutrient agar slants, of pH 7.0. After 24 hr at 37°C, the growth of each slant was washed into 100 ml of nutrient broth, of pH 7.2. The 24 hr broth cultures, tested aerobically and anaerobically for contaminants, were centrifuged, and the supernatants filtered through Mandler candles No. 9. Filtrates, controlled for sterility, were stored at 4°C. Male albino rabbits, weighing about 2 kg, received *E. coli* toxin in saline dilutions ranging from 1:5 to 1:500 in volume of 1 ml per kg of body weight.

Rabbits' aqueous humor, placed into small stoppered tubes, was weighed. The presence or absence of coagulation was noted

* Work supported in part by the John and Mary Markle Fund.

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1 Ayo (Ajo), C., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 500.

2 Ayo, C., accepted for publication by the *Journal of Immunology.*

3 Shwartzman, G., *Phenomenon of Local Tissue Reactivity,* Paul B. Hoeber, N.Y., 1937.

after several hours standing. To each sample, brought to a volume of 1 ml with distilled water, 0.2 ml of 2% gum ghatti and 1 ml of 5% sulphosalicylic acid were added. Tubes were shaken lightly and allowed to stand for several hours. Protein was then determined by either of two methods. When no precipitate was present in the solution, a modification of the Looney-Walsh nephelometric method[4,5] was used: The turbidity was compared with a blank in a photoelectric colorimeter[6] and the galvanometer values were read from a standard curve, calibrated with known serum protein dilutions. When precipitates were present, protein was determined by the Kjeldahl method. After standing overnight in the icebox, the tubes were centrifuged and carefully drained. The precipitate, washed with 2.5% sulphosalicylic acid, was transferred with sulphuric acid into Kjeldahl flasks. A conversion factor of 6.25 was used.

Experiment 1. In the first experiment a comparative study was made of the protein content of the aqueous humor and the previously established ocular symptoms, iridoconjunctival hyperemia and aqueous humor coagulation. Sixty-one rabbits were used. Observation varied from 30 min to 3 hr after toxin injection. In many cases, the protein content did not parallel the dose of toxin; this confirmed the finding[2] that the ocular reaction is considerably influenced by variations in the response of the animals.

Ocular hyperemia was evident when the protein content was above 150 mg %. It was usually proportional to the protein content of the aqueous humor but did not always show a close parallel. Coagulation of the aqueous humor consistently occurred with a protein content of 500 mg % or more, while fibrin threads could be observed with protein concentrations of 200-500 mg %. Red blood cells were never found microscopically even with the highest protein concentrations. Results similar to those obtained in this experiment were recently observed[7] using other Shwartzman toxins.

Experiment 2. In a study of the optimal time of the ocular reaction, 2 groups of 12 rabbits were injected with coli toxin in dilutions 1:5 and 1:50 respectively. Aqueous humor was removed after 30, 60, 90, and 120 min and every hour thereafter up to 12 hr after injection. At every interval one eye each of 2 new rabbits per group was used. In the first group, which received the stronger dose of toxin, protein concentrations were higher, reached a maximum in 2 hr, and started to drop in 6 hr. In the second group the protein content reached a maximum in 3 hr and dropped after 5. Twelve hr after injection the protein content was still elevated in both groups, being 466 and 440 mg % and 190 and 90 mg % respectively.

It was observed in these experiments that the aqueous humor removed more than 3 hr after toxin injection may show fibrin threads or complete coagulation even with protein concentrations of as low as 50-100 mg %. This seems to indicate an earlier decrease of albumin and globulin than of fibrinogen as the reaction subsides.

Experiment 3. The protein content of the secondary aqueous humor was determined in 35 rabbits treated with coli toxin in varying doses. 0.2 ml of aqueous was removed 90 min and 4 hr after toxin injection. As shown in Table I, the animals were divided into 3 groups: Group I those without toxin treatment; Groups II and III including animals injected intravenously with coli toxin, and having a protein concentration in the primary aqueous in the normal limits and above normal respectively. The abnormally high protein content of the secondary aqueous of Group II indicates a toxic effect on the capillaries of the ocular vessels although no primary reaction had been evident. Damage to extraocular structures (paralysis) in the absence of ocular manifestations had been observed previously.[7]

Experiment 4. Nine animals received twice, at 4 weeks interval, the same dose of a filtrate of *E. coli* which proved to be of

[4] Looney, J. M., and Walsh, A. I., *J. Biol. Chem.*, 1939, **127**, 117.

[5] Kronfeld, P. C., *Am. J. Ophth.*, 1941, **24**, 1121.

[6] Weech, A. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 858.

[7] Ayo, C., unpublished experiments.

TABLE I.
Influence of *E. coli* Toxin on Protein Content of Secondary Aqueous Humor.

Group	No. of rabbits	Treatment	Protein content (mg%) Primary Avg	Primary Low	Primary High	Secondary Avg	Secondary Low	Secondary High
I	6	None	36	19	51	565	281	993
II	18	*Coli* toxin	41	16	68	2160	869	2590
III	17	" "	435	77	1025	2211	1297	3800

unchanged potency in control rabbits. Results as shown in Table II confirm the observation[2] that a previous administration of toxin confers a considerable degree of protection against a subsequent one. The nature of this protection is being investigated.

TABLE II.
Protein Content of Aqueous Humor Following Injection and Reinjection (4 Weeks Later) of *E. coli* Toxin.

Toxin dilution	Primary aqueous humor protein (mg%) 90′ after 1st inj.	2nd inj.
1:100	547	431
1:200	1108	97
1:200	1025	55
1:235	77	79
1:240	130	68
1:240*	38	55
1:240	26	70
1:300*	1582	45
1:400	312	48

*Aqueous humor removed 4 hours after 1st and 2nd injections.

Experiment 5. The nature of the proteins in the aqueous humor after injection of coli toxin was studied electrophoretically (Dr. D. H. Moore) by the Tiselius method in a microcell of about 2 ml. Two hr after toxin injection, fluid was removed from the anterior chambers of 8 rabbits. Coagulation of the pooled fluid was prevented by sodium citrate, 6 mg per ml of fluid. The sample contained 353 mg % of protein. It was dialysed at +4°C for 3 days against 0.05 molar barbiturate buffer and 0.1 ionic strength (pH 7.83). The relative concentrations of the proteins after one hr were: albumin 77.5%, beta globulin 11.5%, gamma globulin 6.1%, fibrinogen 4.9%. The A:G ratio was 3.44. A fast component[8] corresponding to hyaluronic acid[9] was not visible, since its concentration was probably too low. The elevated A:G ration seems to indicate a faster diffusion into the anterior chamber of the proteins of low molecular weight.

Summary. Intravenous injection of coli toxin produced in rabbits a rise in protein content of the aqueous humor. Above 500 mg % protein the aqueous coagulated. The protein content of the secondary aqueous of toxin-treated animals was higher than in normal controls, even when that of the primary aqueous had been normal.

A previous dose of coli toxin partially or completely prevented a rise in the protein content after a second injection.

Electrophoresis of the aqueous humor of toxin treated rabbits indicated albumin, beta and gamma globulin, and fibrinogen, with an A:G ratio of 3.44.

8 Meyer, K., and Chaffee, E., *J. Biol. Chem.*, 1940, **133**, 83.

9 Meyer, K., Smyth, E. M., and Gallardo, E., *Am. J. Ophth.*, 1938, **21**, 1083.

13860

Histochemical Reactions for Lipid Aldehydes and Ketones.*

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Bennett[1,2] published a histochemical method for the demonstration of the keto-steroid hormone of the adrenal cortex. The method is based on the formation of a yellow phenylhydrazone on treatment of frozen sections with phenylhydrazine. The reaction is prevented by extraction of the lipids with alcohol or by pretreatment with semicarbazide. From these facts Bennett draws the conclusion that the reaction is due to the presence of a lipid ketone, probably a keto-steroid, "since no aldehydes with the solubility properties of corticosterones have been detected in the adrenal cortex."

Feulgen and his collaborators[3–5] published a series of papers in which they showed the presence in many tissues of a loosely bound aldehyde which they called plasmal. This aldehyde is of lipid nature, and can be liberated from the compound of which it is a component by mild hydrolysis or oxidation. The best reagent for plasmal is Schiff's reagent, fuchsinesulfurous acid, which stains it in an intense purplish shade. Plasmal will also give very promptly all the typical aldehyde reactions, *e.g.*, it forms a bisulfite addition product, a phenylhydrazone, a semicarbazone and a thiosemicarbazone. Further studies showed that plasmal is a rather complex mixture, its bulk consisting of palmitic and stearic aldehydes.

On the basis of these facts Verne[6] worked out a microtechnical method for the demonstration of plasmal in tissue sections, and gave a description of the pictures obtained. Large amounts of plasmal were found in many tissues containing lipids, especially phospholipids. Verne stresses the intense and widespread aldehyde reaction in the adrenal cortex.

As it seemed very probable that Bennett's stain was nothing but a plasmal reaction, the pictures obtained in adrenal glands by the two reactions under various conditions were compared.

The results were the following: oxidation is an important factor in both procedures. Entirely fresh tissues, or tissues fixed for 6 hr in formalin prepared with freshly boiled distilled water and protected from contact with air, do not react visibly either with Schiff's reagent or with phenylhydrazine for several hours. However, both technics prescribe oxidation of the sections before applying the reagent. With the plasmal technic oxidation (or possibly hydrolysis) is catalyzed by mercury bichloride, with Bennett's technic either iodine or aeration in an oxygen-saturated buffer solution is used. During these procedures the acetal linkages break up, and both reactions become positive. Long exposure of the tissues to solutions such as formaldehyde or distilled water, unprotected from air, will have the same effect as direct oxidation. The extent and intensity of the reaction will increase progressively for about 5 to 6 days. The pictures obtained by the two methods were invariably identical, except for the shade. This applies to all species examined (man, dog, cat, guinea pig). As there is a great individual variation in the pattern of the plasmal reaction in human adrenals, it was especially easy to compare the pictures in a considerable detail.

* This work has been supported by grants from the Douglas Smith Foundation for Medical Research of the University of Chicago and from the Committee on Scientific Research of the American Medical Association.

1 Bennett, S. H., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 786.

2 Bennett, S. H., *Am. J. Anat.*, 1940, **67**, 151.

3 Feulgen, R., and Voit, K., *Arch. ges. Physiol.*, 1924, **206**, 389.

4 Imhäuser, K., *Biochem. Z.*, 1927, **186**, 360.

5 Feulgen, R., and Behrens, M., *Z. physiol. Chemie*, 1928, **256**, 15.

6 Verne, J., *Ann. de physiol.*, 1929, **5**, 245.

Moreover, sections from different kinds of necrobiotic tissue (tubercles, infarcts, tumors) which show a very intense plasmal reaction were equally positive with Bennett's stain, and the pattern of distribution shown by the two reactions was again identical. The same applies to myelin sheaths both in the brain and in peripheral nerves, although neither necrotic tissues nor myelin are known to be rich sources of ketosteroid hormones.

While ketosteroids under the conditions of Bennett's reaction do give phenylhydrazones similar to that of plasmal, the method is by no means specific for them, the less so as even a positive ketosteroid reaction would be completely masked by the incomparably more intense plasmal reaction. Only a ketone test which is directly positive without any previous oxidative treatment of the tissues can be accepted as a presumptive evidence for the presence of ketosteroids, if the possibility of a false positive reaction due to hydrolysis of plasmal can be ruled out.

Summary. Bennett's histochemical test for ketosteroids in the adrenal cortex is not a specific reaction since it merely indicates the presence of lipid bodies having aldehyde or keto groups. Lipid aldehydes, not related in any way to ketosteroids, are present in large amounts in the adrenal cortex.

13861

Absorption of Carbohydrates in Humans.*

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The rate of absorption of glucose from the intestinal tract has been generally considered to be independent of both the concentration of glucose, and the absolute amount of glucose present in the intestine. However, observations on the absorption of various hexoses have revealed that the rate of absorption varies in the following order: galactose>glucose>fructose>mannose>xylose>arabinose.[1] The investigation by Cori was conducted on rats and the technic consisted of the oral administration of various carbohydrates in various dosages. After definite intervals the animals were sacrificed and the carbohydrate remaining in the intestine was determined.

In our own studies on insulin shock treatment, it was observed that the time required for the patient to rouse from coma was dependent on the concentration of glucose administered. In the ordinary procedure for insulin shock treatment, patients are awakened by the oral administration of 600 cc of 30% glucose, and they regain consciousness in about 15 to 30 min. Frequently patients failed to rouse from coma with 30% glucose and required the administration of glucose intravenously. In such patients, almost invariably, the administration of 600 cc of 5% glucose terminated the coma within a reasonable time. It was therefore thought of interest to determine the rate of absorption of glucose solutions varying from 5 to 30% concentrations.

Method. Patients were chosen from the wards of Bellevue Psychiatric Hospital, who were to all intents and purposes physically normal. The diagnoses of these cases included psychopathic personality and recovered reactive depressions in whom the situational defect causing the difficulty had been alleviated while the patient was in the hospital. On the morning of the experiment, patients received no breakfast and after a one-half hour rest in bed, a fasting blood sample was drawn from the antecubital vein. A 30 g dose of glucose in concentrations varying from 5 to 30% was administered by mouth and blood samples were obtained at intervals of 10, 15, 30, and 60 min. Blood sugars were

* Aided by a grant from the William R. Warner & Company Fellowship Fund.

[1] Cori, C. F., *J. Biol. Chem.*, 1925, **66**, 691.

analyzed by the Wendel[2] modification of the Hagedorn and Jensen method.

With the second group of patients similar studies were made in which the total volume of fluid was kept constant and the concentration of glucose administered varied from 3 to 30%. Fasting blood samples were taken after a one-half hour rest in bed, and 200 cc of the glucose solution was administered by mouth. Blood samples were obtained subsequently at 10, 15, 30, and 60 min.

Results. The changes in blood sugar after the administration of 30 g of glucose in concentrations varied from 5 to 30% and are presented in Table I. It may be seen that the blood sugar rose in 10 min only with the 5 and 10% concentrations while the administration of 15 or 30% glucose showed no change. The administration of 600 cc of 5% glucose produced a maximum rise in the blood sugar in 30 min, and the rate at which the blood sugar rose exceeded the other concentrations. After the administration of 10% glucose the peak of the blood sugar did not occur within one hour, and the rise in blood sugar exceeded that observed with 15 and 30% glucose. There appeared to be no appreciable difference between the blood sugar curves after the administration of 15 and 30% concentrations. Data on experiments in which the total volume of solution administered was kept constant and the concentration varied from 3 to 30% are presented in Table II. Despite the fact that the dose varied from 60 g in the 30% concentration while only 6 g of glucose was ingested in the 3% concentration, there was no appreciable difference in the rise of the blood curve during the first 30 min at any of these concentrations. However, the one-hour specimen shows a

TABLE I.
Change in Blood Glucose After Oral Administration of Glucose (mg%)

Pt.	10 min	15 min	30 min	60 min
	Dose—5% (600 cc) 30 g.			
1	9	9	36	37
2	22	29	45	4
3	16	38	67	41
4	5	31	45	35
5	9	25	43	22
6	26	33	36	24
7	12	48	67	70
	—	—	—	—
Avg	14	30	48	33
	Dose—10% (300 cc) 30 g.			
1	11	16	49	64
2	17	24	40	69
3	3	13	57	48
4	10	18	39	40
5	0	15	30	53
6	12	20	60	64
	—	—	—	—
Avg	9	18	46	56
	Dose—15% (200 cc) 30 g.			
1	7	3	11	
2	—2	3	22	
3	—8	9	14	
4	4	17	27	32
5	—2	1	44	19
6	—2	0	36	70
7	5	7	36	24
	—	—	—	—
Avg	0	6	27	36
	Dose—30% (100 cc) 30 g.			
1	—4	—6	22	13
2	5	10	25	15
3	—3	6	10	24
4	6	5	23	29
5	1	3	18	49
	—	—	—	—
Avg	1	4	20	26

TABLE II.
Change in Blood Glucose After Oral Administration of Glucose.

Pt.	10 min	15 min	30 min	60 min
	Dose—3% (200 cc) 6 g.			
1	7	19	25	
2	10	13	12	
3	3	3		
	—	—	—	
Avg	7	12	19	
	Dose—5% (200 cc) 10 g.			
1	5	8	24	24
2	2	15	19	—1
3	14	28	33	29
4	—4	5	22	5
5	1	4	21	—2
6	16	35	31	0
7	7	16	24	33
	—	—	—	—
Avg	6	14	25	13
	Dose—15% (200 cc) 30 g.			
1	7	3	11	
2	—2	3	22	
3	—8	9	14	
4	4	17	27	34
5	—2	1	44	29
6	—2	0	36	70
7	5	7	36	24
	—	—	—	—
Avg	0	6	27	39
	Dose—30% (200 cc) 60 g.			
1	9	15	26	18
2	—3	7	33	48
3	4	3	32	37
4	10	10	9	63
	—	—	—	—
Avg	5	8.5	25	42

[2] Wendel, W. B., *J. Biol. Chem.*, 1933, **102**, 47.

definite tendency toward a higher blood sugar with a greater dosage of glucose.

Discussion. The disposal of sugar in the animal body is dependent on 4 distinct processes: first, the absorption from the intestinal tract; second, the passage through the blood and its elimination in the urine; third, penetration through the tissues; and fourth, its synthesis by the tissues. There have been numerous investigations on the absorption of sugar from the intestinal tract and its passage into the blood stream. Cori[1] investigated the disappearance of glucose from the intestinal tract of rats by means of sacrifice experiments in which the amounts of glucose remaining in the intestinal tract at definite periods were analyzed. In his experiments glucose was administered in 25, 50, and 80% solution and he observed no difference in the rate of absorption of these concentrations. Similar observations on galactose, fructose and mannose revealed that the absorption of these sugars was also independent of these high concentrations in the intestine. Previous investigations on the absorption of sugars from the intestinal tract were directed towards a comparison of the various hexoses and no attention was paid to the concentration of the sugar.[3,4]

These observations are contrary to our own clinical observations on the effects of various concentrations of glucose on the time required to rouse patients from the insulin shock. The data reported in the present paper also indicated that glucose is more rapidly absorbed in the 5% solution than in the 30% solution. Since one factor involved in absorption through a semi-permeable membrane is the equilibrium established between the fluid in the intestine and the blood stream, it would seem on an *a priori* basis that differences would be observed in the rate of absorption with different concentrations of glucose in the intestine. This effect has been investigated in the animal by Darrow and Yannet[5] and Gilman.[6] These investigators found in experiments on dogs, rabbits, and monkeys that the injection of 5% glucose solution intraperitoneally resulted in the establishment of an equilibrium between the peritoneal fluid and the blood serum before absorption actually took place. During the initial period of several hours, glucose molecules passed into the blood stream while electrolytes appeared in the peritoneal fluid, and only after a considerable period was there an actual disappearance of fluid from the peritoneal cavity. In a typical example which Darrow and Yannet report in Table I on page 267, 500 cc of glucose was injected and after 6 hr they could recover 467 cc. There was a fall in serum sodium and chloride which appeared in the peritoneal fluid.

There is some indication in Cori's article that such a process may well take place in the intestinal tract. On page 706, he reports, "It was noted that the rats showed a greatly distended stomach when examined 1 to 3 hr after sugar feeding. This suggested that the stomach water was attracted by a hypertonic solution." In view of the observations of Darrow and Yannet, it seems unlikely that the only process was the attraction of the water due to the hypertonic solution but rather that there was an attempt to equilibrate the intestinal solution with the blood serum, which would involve the passage of electrolytes into the stomach and intestines with the fluid.

Summary and Conclusions. We have presented blood sugar curves in human patients following the oral administration of glucose solutions varying from 5 to 30%. The initial rise in blood sugar during the first 30 min was more rapid with the lower concentration. It was also noted that the administration of 5% glucose solution to patients in insulin coma produced a more rapid arousal from the coma than with the 30% ordinarily employed. These results are in contrast to earlier work indicating that the absorption of glucose was independent of its concentration in the intestine. This evidence supports the hypothesis that the absorption from the intestine is at least partially dependent on the establishment of an equilibrium between the intestinal solution and the blood serum.

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3 Nagano, J., *Arch. Geo. Physiol.*, 1902, **90**, 389.

4 Hewitt, A., *Biochem. J.*, 1924, **18**, 161.

5 Darrow, D. C., and Yannet, H., *J. Clin. Invest.*, 1935, **14**, 266.

6 Gilman, A., *Am. J. Physiol.*, 1934, **108**, 662.

13862 P

Electrophoretic Analysis of Extracts of Rabbit Papillomas.*

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The serum of rabbits bearing the growths of infectious papillomatosis and of rabbits immunized with purified virus or wart extracts has been studied[1] by the electrophoretic procedures of Tiselius. Differences from the normal electrophoretic patterns were seen only in the serum of animals actually carrying growths. In the present work studies were made on saline extracts of the virus-induced growths of domestic and cottontail rabbits.

The growths had been present for 39 to 115 days and, except for retrogressing warts in one animal, were exuberant and fleshy. Growths were removed from rabbits killed by air injection and, freed of connective tissue, were pulped in a Waring Blender and extracted for 24 hr at 2-8°C in 20% suspension in 0.9% NaCl solution. The extracts were filtered with celite and dialyzed against buffer prepared as in the serum studies.[1]

In Fig. 1 is shown the electrophoretic diagram of a filtered extract of domestic rabbit papillomas showing 3 distinct migrating peaks, the mobilities of which were 5.73, 3.07, and 1.22 cm^2/sec. volt, respectively. These values corresponded fairly closely to the mean mobilities found for albumin, beta globulin and gamma globulin of normal rabbit serum,[2] namely 5.13, 2.98 and 0.92, respectively. The greatest difference was seen with the peak in the relative position of the gamma globulin of serum diagrams. It has been noted that when filtered extracts are heated to 56°C for 30 min some of the protein is coagulated, a phenomenon not observed when serum is so heated. The diagram obtained with extract of Fig. 1 after being heated in this way is shown in Fig. 2. The protein coagulated and removed was 43% of the total present in the unheated extract. In Fig. 2 are seen again 3 chief components, the mobilities of which were, 5.20, 2.78, and 1.04 cm^2/sec. volt.

These findings suggest that a large portion of the protein of the domestic rabbit papilloma extracts may be very similar to, if not identical with, the protein of rabbit serum. That much of it differed from serum protein was indicated by the amount coagulated at 56°C for 30 min. After heating, the residue of the 3 components, designated as A, B, and C, resembled more closely in mobility the albumin, beta globulin and gamma globulin components of serum. The serum of the animal furnishing the growths of Fig. 1 contained albumin plus alpha globulin 55.1%, beta globulin 30.3%, and gamma globulin 14.6%. In the unheated extract the percentage composition was 28.7%, 39.8% and 31.5% for the A, B, and C components, respectively. For the heated extract, the values were 48.0%, 30.1% and 21.9%, a distribution more closely approximating that of the albumin, beta globulin and gamma globulin of serum. The protein loss due to heat was slight for the A component, 3.9%, most of it being distributed between the B, 57%, and C, 60%, components.

Extracts from cottontail rabbit growths differed greatly from those of domestic rabbit growths both in composition and behavior. Again 3 peaks were noted in the diagrams of the filtered extracts, an example of which showed a percentage composition with respect to the A, B, and C components of 37.8%, 41.0% and 21.2%. Heating resulted in the removal of only 10% of the total protein distributed approximately equally among the A, B, and C components.

Further work is necessary to establish the

* This work was aided by grants from the Lederle Laboratories, Inc., Pearl River, New York, and by the Dorothy Beard Research Fund.

1 Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 358.

2 Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Immunol.*, 1942, **44**, 115.

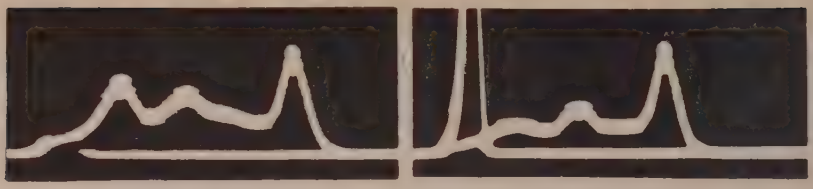

FIG. 1. Electrophoretic diagram of a filtered extract of domestic rabbit papillomas. The curve was photographed after migration for 206 minutes. The potential gradient was 4.66 volts/cm at 0.2 ionic strength and 1°C.

FIG. 2. Diagram obtained after heating extract of Fig. 1 at 56°C for 30 minutes. The curve was photographed after migration for 0 and 176 minutes. The potential gradient was 4.78 volts/cm at 0.2 ionic strength and 1°C.

origin and nature of the proteins in the papilloma extracts. No evidence was seen of a component behaving electrophoretically as the papilloma virus.[3] The protein coagulated at 56°C for 30 min was probably of tissue origin though it may have been altered serum protein. After heating, the protein of the extract still differed in its distribution among the 3 components from the analogous distribution in serum between albumin, beta globulin and gamma globulin. From hemoglobin determinations the wart tissue extract of Fig. 1 and 2 did not contain more than 0.049 cc of whole blood per g of warts extracted. If the protein of the heated extract is assumed to be of serum origin, the amount present per g of warts would represent 0.45 cc of serum. It is possible that some tissue protein indistinguishable from serum protein electrophoretically remained after heating, an indication of which was seen in the distribution among the A, B, and C components. From calculations based on the assumption of the identity of the A component with serum albumin, the equivalent amount of serum possibly present was 0.39 cc per g of warts. In the instance of cottontail rabbit warts similar calculations gave values of 0.91 cc and 0.68 cc per g of warts.

The data demonstrate the presence of protein in rabbit papilloma extracts similar in electrophoretic behavior to the protein of serum and provide an estimate of its quantity. The evidence indicated that the protein was located in the wart substance which it probably entered during the period of growth. The papilloma is virus-induced and the serum of animals carrying it contains virus immune bodies. It is possible that serum immune bodies might exert an influence either on the growth itself or on the virus present in the growth.[4] No evidence for this was seen since more protein was found in fleshy warts than in those growing less well. Further, contrary to expected results, more serum protein was found in cottontail rabbit warts which generally yield much virus than in domestic rabbit growths which seldom yield active virus.

[3] Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1942, **142**, 193.

[4] Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.

13863

Destruction of Sulfonamide Inhibition Present in Sera by Soil Bacillus (Mirick)*

DANIEL A. BOROFF AND JESSE G. M. BULLOWA.

From the Harlem Hospital and the Littauer Pneumonia Research Fund of New York University College of Medicine.

Mirick[1] described a Gram negative soil bacillus which was capable of oxidizing 10 mg of *p*-aminobenzoic acid in 100 ml of medium in 24 hr. Because *p*-aminobenzoic acid strongly inhibits the action of sulfapyridine, it was deemed important to determine whether the bacillus would destroy the inhibitor found in some human serums.

A culture of this organism was generously supplied by Doctor George S. Mirick, and was tested against several human serums which inhibited sulfapyridine and the highest dilution in which they reacted against 5 mg of sulfapyridine per 100 ml was determined. Previous experiments showed that 0.2 to 0.3 mg % of *p*-aminobenzoic acid is necessary to inactivate 5 mg % of sulfapyridine, so that the concentration of inhibitors may be translated into mg % of *p*-aminobenzoic acid activity.

Tubes containing inhibitor free beef heart infusion broth and varied amounts of inhibiting serum with *p*-aminobenzoic acid were set up. One set of tubes seeded with 0.5 ml of an 18-hr culture of the soil bacillus culture was incubated over night at 37°C. Next morning the tubes containing the soil bacillus as well as a similar set of sterile tubes were heated for 2 hr at 56°C. The dead organisms were removed by rapid centrifugation. All the tubes were then seeded with an 18-hr culture of our standard pneumo-

TABLE I.
Effect of Soil Bacillus (Mirick) on Human Serum Inhibition of Sulfapyridine 5 mg per 100 cc.

No.	Sulfapyridine 5 mg% and serum	Inoculated with soil bacillus	Growth of pneumococcus T 3-1	
			24 hrs	48 hrs
1	M	+	±	0
2	M	0	++++	++++
3	C	+	±	0
4	C	0	++++	++++
5	S	+	±	++++
6	S	0	++++	++++
7	D	+	+	++++
8	D	0	++++	++++
9	Kle*	+	±	0
10	Kle*	0	0	0
11	Kau*	+	0	0
12	Kau*	0	0	0
13	Broth + 3 mg% PABA	+	±	0
14	Broth + 3 mg% PABA	0	++++	++++
15	Broth + 0.2 ml horse serum	+	0	0
16	Broth + 0.2 ml horse serum	0	0	0

The initial inoculum of Pneumococcus T 3-1 was 1000 org/ml.
*Serums Kle and Kau contained no inhibitors and were included as controls.
+ indicates that the media was inoculated with soil bacillus.
0—tube was not inoculated.
PABA—*p*-aminobenzoic acid.
± cloudiness, small amount of growth present.
++++ abundant growth.

* This study received additional financial support from the Metropolitan Life Insurance Company, New York City, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch and Mrs. H. Robert Samstag.

[1] Mirick, G. S., *J. Clin. Invest.*, 1941, **20**, 434.

coccus III to contain 1000 organisms per ml. Each tube also received enough sulfapyridine to make its concentration 5 mg % and all were incubated over night at 37°C. The table shows that sulfonamide inhibiting action was removed from serum by the soil bacillus.

Conclusion. It may be inferred from these experiments that soil bacillus (Mirick) interferes with the activity of the substance in serum which inhibits sulfapyridine.

13864 P

Experimental Transmission of *Lymphogranuloma venereum* Virus Through the Placenta.

HUGO HELLENDALL. (Introduced by James W. Jobling.)

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

In view of the recent emphasis by Goodpasture[1] on transmission of viral infections to the fetus, and because of the possibility of applying experimental study to human cases with eventually a clearer understanding of virus pathogenesis, an investigation dealing with the experimental transmission of the lymphogranuloma virus through the placenta of mice was undertaken. This animal host was selected because of its well-known susceptibility by intracerebral route to the agent under investigation. Two lines of study were followed: (1) injection of pregnant mice by non-infecting routes and (2) infection of the host by the usual experimental portal of entry, the brain. In both types of experiments a fixed mouse strain of lymphogranuloma virus was injected in dilutions of 1:10 into pregnant mice. These animals were sacrificed 24, 48, 72 and 96 hr after the injections, or after they had gone to term and kindled. At these periods, emulsions from the brains of both mother and fetus were injected into other mice. Part of the material so injected was made up into antigens in the usual manner (heating 3 hr at 58°). The antigens were then injected intradermally into human beings and compared with human antigen or Lygranum.[1] As has been recommended,[2,3] a skin test was considered positive if a papule with a diameter of 5 mm appeared within 48 hr. Normal mouse brains (maternal and fetal) treated similarly to the antigens were used as controls. Fifty-three human cases were tested. The presence or absence of virus in fetal tissue was also studied by intracerebral injections of tissue emulsions into normal mice.

Human and mouse antigens in 37 cases with positive Frei tests (human antigen) gave the following results (Table I). In 30, fetal brain antigens produced positive skin reactions. In 12 negative cases, the same type of antigen yielded 10 negative skin tests. The table shows also strong reactions to antigens prepared from the brains of infected mothers (11 positive and 9 indefinite). Again, in agreement with previous investigators,[4] the limitations of the use of mouse brain antigens are apparent by the number of non-specific reactions attendant upon injection of such animal tissue into the human skin.

In one experiment 96 hr after subcutaneous injection of virus, the mother was sacrificed and fetal brain emulsion was inoculated intracerebrally into 4 normal mice. Seven days post-inoculation all were sick but the illness did not progress and the ani-

1 Goodpasture, Ernest W., *Science*, 1942, **95**, 391.

2 Ormsby, Oliver S., *A Practical Treatise on Diseases of the Skin*, Lea and Febiger, Philadelphia, 1937, 978.

3 Harvard School of Public Health Symposium, *Virus and Rickettsial Diseases*, Harvard Univ. Press, Cambridge, Mass., 1940, **372**, 371.

4 Reider, Reuben Frank, and Cañizares, Orlando, *Arch. Dermat. and Syph.*, 1938, **38**, 930.

TABLE I.
Comparison of Human and Mouse Antigens in Humans.

Frei test with human antigen	Mouse brain antigens					
	Positive		Indefinite		Negative	
	Maternal	Fetal	Maternal	Fetal	Maternal	Fetal
37 +	11	30	9	4	9	3
4 ?	—	1	3	1	1	2
12 —	—	—	4	2	4	10

mals recovered. In a second subcutaneous experiment, mother and fetal brain emulsions were similarly injected into 34 mice. Of the group receiving maternal brain emulsions, 8 showed symptoms, with death occurring in 5 animals. In the case of the mice injected with fetal brain emulsions, 6 showed symptoms and deaths occurred in 3 of the animals. To determine the ease with which the agent could be transmitted in this fashion, one of the mice showing symptoms after injection with fetal brain emulsion was sacrificed. Its brain further transmitted to 4 mice produced characteristic symptoms and death in all animals. On routine examination no bacterial contaminations were observed.

To further determine the specificity of the agent transmitted from fetus brains to adult mice, emulsions of fetal tissue were made into antigens and tested in known human cases of lymphogranuloma. When compared with human antigen there seemed no reason to doubt that the virus of *Lymphogranuloma venereum* had been present in the brains of these animals (Table II).

Results similar to those recorded in Table II were obtained when pregnant mice were injected intracerebrally instead of subcutaneously.

TABLE II.
Comparison of Human and Mouse Antigens in Humans.

Frei test with human antigen	Fetal antigen	Mouse brain antigens. Antigen from adult which received fetal brain emulsion
+	+	+
+	+	+
+	+ (weak)	+
+	—	+
+ (weak)	—	+
+	not done	+

Furthermore, in touch preparations from the brains of various fetuses after maternal injection with *Lymphogranuloma venereum* virus, bodies morphologically similar to elementary bodies of lymphogranuloma have been noted, after staining with Giemsa.

On the basis of the investigations herewith reported, the conclusion appears justified that the lymphogranuloma virus is transmitted in pregnant *mice* via placenta into the fetus.

I wish to express my gratitude to Dr. Murray Sanders, Department of Bacteriology, Columbia University, for the strain of Lymphogranuloma virus, also for advice and assistance given during the progress of the work.

13865 P

Culex tarsalis Coq. a Proven Vector of St. Louis Encephalitis.*

W. McD. Hammon and W. C. Reeves. (Introduced by K. F. Meyer.)

From the George Williams Hooper Foundation for Medical Research, University of California, San Francisco, and the Texas State Health Department, Bureau of Laboratories, Austin, Texas.

During the summer season of 1941 in the Yakima Valley, Washington, *Culex tarsalis* Coq. was thrice found infected with the virus of St. Louis encephalitis.[1] In addition the seasonal activities and feeding habits of this species were found to fit the epidemiological picture of the infection in that area.[2] Previously *Culex* mosquitoes had been suspected in the 1933 St. Louis outbreak.[3] In support of this latter observation *Culex pipiens* Linn. was found capable of transmitting the virus by Mitamura and associates,[4] and recently their results were confirmed by Reeves, Hammon and Izumi.[5]

The experimental data presented in this paper complete the evidence incriminating *C. tarsalis* as a vector of St. Louis virus, for it may now be reported that this species which was found to serve as a host in nature is also a good experimental vector.

Experiments. Two experiments were carried out in a temporary field laboratory established in San Benito, Texas. All mosquitoes used were raised from larvæ. The virus employed was strain F-103, only 3 mouse brain passages removed from that isolated by mouse inoculation from *C. tarsalis* taken in the field.[1] The chickens used as experimental hosts were raised from the incubator in a room entirely free from mosquitoes and flies.

On April 26th, 1942 two *C. tarsalis* were fed on cotton soaked with a blood-virus mixture. After 8 days incubation at laboratory temperatures varying from 22° to 38°C these same 2 mosquitoes fed on a normal 2 months-old chicken. The serum of this chicken 48 hr later contained St. Louis virus as demonstrated by intracerebral inoculation of mice. A neutralization test against the St. Louis virus with serum from the same chicken taken 20 days later indicated that antibodies had formed. The mosquitoes refused to feed again so were killed after a total of 15 days incubation. Virus was then isolated from a suspension of the two, by mouse inoculation.

On May 5th, 1942, fifty-two *C. tarsalis* were similarly given a blood-virus meal. Attempts were made to isolate virus from the serum of a series of 2-months-old chickens on which the mosquitoes had fed. Each time the mosquitoes were permitted to feed a different chicken was employed. Eight mosquitoes fed after 4 days' incubation, 15 after 6 days, 5 after 8 days, and 6 after 10 days. Virus was isolated 24 and 48 hr after the mosquitoes had fed from the serum of those chickens used on the 4th, 6th and 10th days, but not on the 8th day. Neutralization tests performed on sera taken at least 20 days later indicated that all chickens had been infected. Only 18 mosquitoes were alive on the 10th day. These were killed

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Hammon, W. McD., Reeves, W. C., Brookman, B., Izumi, E. M., and Gjullin, C. M., *Science*, 1941, **94**, 328; Hammon, W. McD., Reeves, W. C., Brookman, B., and Izumi, E. M., *J. Infect. Dis.*, 1942, **70**, 263.

2 Hammon, W. McD., Reeves, W. C., Brookman, B., and Gjullin, C. M., *J. Infect. Dis.*, 1942, **70**, 278; Hammon, W. McD., Gray, J. A., Evans, F. C., Izumi, E. M., and Lundy, H. W., *Science*, 1941, **94**, 305; Hammon, W. McD., Lundy, H. W., Gray, J. A., Evans, F. C., Bang, F., and Izumi, E. M., *J. Immunol.*, 1942, **43**, 75; Bang, F., and Reeves, W. C., *J. Infect. Dis.*, 1942, **70**, 273.

3 Lumsden, L. L., unpublished official report, 1933.

4 Mitamura, T., Yamada, S., Hazato, H., Mori, K., Hosoi, T., Kitaoka, M., Watanabe, S., Okubo, K., and Tenjin, S., *Tr. Jap. Path. Soc.*, 1937, **27**, 573.

5 Reeves, W. C., Hammon, W. McD., and Izumi, E. M., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 125.

and from them virus was isolated.

Since the number of available *C. tarsalis* was very limited no attempt was made in these preliminary experiments to infect them by feeding on an experimental animal, for in the laboratory they feed much more satisfactorily on blood-soaked cotton. However, we have infected other mosquitoes by permitting them to feed on a chicken 48 hr after a subcutaneous inoculation of the St. Louis virus. Finding *C. tarsalis* infected in nature is evidence that it becomes infected by some natural means, probably by feeding on an infected animal host.

In addition to *C. tarsalis, Culex coronator* Beyer was shown to be capable of transmitting the St. Louis virus to chickens. This latter species is potentially important in the lower Rio Grande Valley, where the work was done. In this area during an epidemic of encephalitis in 1941 antibodies to the St. Louis virus were found in the sera of man and domestic animals.[6]

Summary and Conclusions. *Culex tarsalis* has been demonstrated to be infected in nature with St. Louis encephalitis virus and to be capable of transmitting it. Furthermore, it fits well into the epidemiological picture encountered in the Yakima Valley, Washington. We can therefore safely conclude that this species is a natural vector. This is the first instance in which a mosquito has fulfilled these 3 criteria for incrimination as a vector in respect to St. Louis encephalitis virus. *C. pipiens* previously has been shown to be capable of transmitting this virus in the laboratory and the same has just been demonstrated for *C. coronator*. In addition, these experiments demonstrate that the chicken may serve as a satisfactory reservoir of virus. This is undoubtedly shared by other birds for we have previously shown that virus may be isolated from the blood of a dove following the bite of infected *C. pipiens*[5] and unpublished work indicates that ducks may serve as reservoirs.

Collection of the larvæ for the second experiment was made by Mr. R. B. Eads, entomologist, Texas State Health Department. Valuable laboratory assistance was rendered by Mrs. Margaret Gray and Miss Margaret Smith, Hooper Foundation.

6 Hammon, W. McD., *J. A. M. A.*, in press. (Paper read at the Convention of the American Medical Association, June, 1942.)

13866

Influence of Diet on Action of the Sulfonamide Drugs.*

ESTHER M. GREISHEIMER, ROBERTA HAFKESBRING AND GRACE E. WERTENBERGER.
From the Department of Physiology, Woman's Medical College of Pennsylvania, Philadelphia.

The importance of diet in the normal physiology of the liver and in the protection of the liver from the action of such damaging substances as chloroform and carbon tetrachloride has been brought to the attention by many investigators.[1-7]

Since we found that sodium sulfapyridine exerts a definite and characteristic effect on the levels of blood sugar and liver glycogen,[8] we wished to study the effects of various diets on the action of sodium sulfapyridine and sodium sulfathiazole in rats. The diets used to date are Purina Dog Chow Checkers

* Supported by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

1 Goldschmidt, S., Vars, H. M., and Ravdin, I. S., *J. Clin. Invest.*, 1939, **18**, 277.

2 Ravdin, I. S., *Am. J. Surg.*, 1938, **40**, 171.

3 Ravdin, I. S., Rhoads, J. E., Frazier, W. D., and Ulin, A. W., *Surgery*, 1938, **3**, 805.

4 Greene, C. H., and Farrell, E., *Arch. Int. Med.*, 1940, **65**, 847.

5 Greene, C. H., and Hotz, R., *Arch. Int. Med.*, 1939, **63**, 778.

6 Channon, H. J., *Ann. Rev. Biochem.*, 1940, **9**, 231.

7 Connor, C. L., *J. A. M. A.*, 1939, **112**, 387.

8 Greisheimer, E. M., Hafkesbring, R., and Magalhaes, H., *Med. Times*, 1941, **69**, 170.

TABLE I.
Effects of Various Diets on Action of Sodium Sulfapyridine and Sodium Sulfathiazole.

Diet	1	2	3	4	5	6	7	8	9	10	11	12	13	Condition
Purina	5	138.6	6.08	4.39	12.4	14.4	+18	90	3.67	161.3	1.00	1.00	0	Controls
Dog Chow	13	139.0	5.55	3.99	12.8	13.2	+26	116	1.22	51.8	33.95	41.91	5.97	Sodium Sulfapyridine
Checkers	10	135.8	5.92	4.36	11.0	13.2	+23	97	3.19	141.4	11.08	13.04	1.96	Sodium Sulfathiazole
High	4	116.0	5.26	4.53	5.7	4.0	— 6.2	96	1.64	76.0	1.36	1.36	0	Controls
Protein	10	118.4	5.46	4.61	6.6	6.4	— 0.2	191	1.11	53.2	46.24	52.55	6.31	Sodium Sulfapyridine
High Carbohydrate	3	113.7	5.69	5.00	8.6	6.7	— 1.7	88	6.70	341.5	1.22	1.22	0	Controls
	9	114.8	4.81	4.19	8.8	7.3	+ 2.1	137	1.58	68.9	54.75	60.32	5.56	Sodium Sulfapyridine
High Fat	4	131.0	6.22	4.75	5.7	4.5	+ 6.5	76	1.23	57.6	1.50	1.50	0	Controls
	4	139.5	5.97	4.28	5.4	4.7	+ 1.2	107	1.22	52.1	32.09	36.82	4.73	Sodium Sulfathiazole

1. No. of rats.
2. Body wt.
3. Liver wt.
4. Liver as % of body wt.
5. Avg daily intake (g).
6. Intake during last 24 hr.
7. Gain or loss in body wt during last week.
8. Blood sugar, mg%.
9. Glycogen, % of liver wt.
10. Glycogen, mg per 100 g body wt.
11. Concentration of free drug in blood, mg%.
12. Concentration of total drug in blood, mg%.
13. Concentration of drug in combined form, mg%.

as the balanced diet, and experimental diets in which protein, carbohydrate and fat form about 87% of the caloric value of the diet.[9]

In these studies the rats were kept on the special diets for one week, and were not fasted before the experiment. The daily food intake and gain or loss in body weight during the last week were recorded. This information, together with the blood sugar, liver glycogen (expressed both as per cent of liver weight and as mg per 100 g of body weight), free, total and acetylated levels of the drug in the blood is shown in Table 1.

One dose of the drug to be studied was given by intraperitoneal injection. 7.5% solutions were used; 1 cc per 100 g of body weight was given. The animals were killed 3 hr later.

It will be noticed that the administration of sodium sulfapyridine to rats that have been on the balanced diet leads to a decided decrease in liver weight, an increase in blood sugar and a decrease of 68% in liver glycogen (calculated from mg of liver glycogen per 100 g of body weight). The changes which follow the administration of sodium sulfathiazole are in the same direction but less marked; the level of the drug in the blood was lower in this group.

On the high protein diet the livers of the control rats were heavier than on the balanced diet. The liver weight increased after the administration of sodium sulfapyridine so these livers were much heavier than the corresponding livers on the balanced diet. The increase in blood sugar was much greater although the values in the control rats on the two diets are almost the same. The most significant finding is the less marked decrease in liver glycogen. The decrease after administration of sodium sulfapyridine is 30% on the high protein diet compared with 68% on the balanced diet, although the level of the drug in the blood is higher in the former rats. This suggests that with sodium sulfapyridine there is less interference with the factors which control liver glycogen if rats have been fed a high protein diet during the preceding week. Studies are in progress with other concentrations of sodium sulfapyridine.

The heaviest livers were found in rats on the high carbohydrate diet. After the administration of sodium sulfapyridine there was a decrease in liver weight and an increase in blood sugar. Although the level of liver glycogen in the control rats is much higher than in the control rats on the balanced diet, the decrease of 79% is greater than that of 68% found on the balanced diet.

The effects of a high fat diet on the action of sodium sulfapyridine have not yet been studied. A few observations on sodium sulfathiazole have been made. The administration of this drug leads to a decrease in liver

[9] Greisheimer, E. M., and Johnson, O. H., *J. Nutrition*, 1930, **3**, 297.

weight and an increase in blood sugar; these changes are more marked than they were on the balanced diet; the level of the drug in the blood is higher. The decrease in liver glycogen is of the same order as on the balanced diet.

In conclusion, it appears that a high protein diet decreases the effect of sodium sulfapyridine on liver glycogen, while a high fat diet does not appear to increase the susceptibility of the liver to the action of sodium sulfathiazole.

13867

Sulfonamides and the Blood pH.*

GRACE E. WERTENBERGER. (Introduced by E. M. Greisheimer.)

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In a previous paper (Wertenberger[1]) on the effect of single doses of some of the sulfonamide preparations upon blood pH, it was shown that intraperitoneal injections of the sodium salts of sulfapyridine (sodium 2-para-amino-benzene-sulfonamido pyridine) and sulfathiazole (sodium 2-para-amino-benzene-sulfonamido-thiazole) produced a marked rise in blood pH, while sulfadiazine (2-sulfanil-amido-pyrimidine) showed only a slight rise.

Since the sulfonamides are most frequently given by mouth, this study was extended to include a comparison of the effects produced by oral and intraperitoneal administration.

Method. Albino male rats from a standard strain of approximately the same age and weighing between 100 and 150 g were used. The animals were kept on a standard diet (Purina Dog Chow Checkers) for one week before treatment. All rats were fasted for 18 hours before the experiment.

The blood pH was determined electrometrically with the glass electrode using the Behrmann-Fay[2] blood chamber as previously described (Wertenberger[1]).

Drug concentrations in the blood were determined by the method of Bratton and Marshall[3] using the photoelectric colorimeter.

A sufficient amount of blood was withdrawn at one time for both the pH and drug concentration determinations in all of the experiments except two.

Results and Discussion. A total of 147 rats was used for this study and the data are summarized in Table I. Sodium sulfapyridine and sodium sulfathiazole produce a marked rise in blood pH when given intraperitoneally and the blood level is high.

This shift does not occur, however, when the drugs are given orally and the blood level is much lower. In the series run 6 hours after treatment, there was a slight rise in the blood pH with sulfapyridine but none with sulfathiazole even though the concentration in the blood had risen to about one-third of the concentration observed after intraperitoneal injection.

The effect of sulfathiazole is not quite as marked, however, and the blood levels are lower and show the least conjugation. This latter fact was observed by Van Dyke and co-workers[4] who found that sulfathiazole is more rapidly metabolized and undergoes less conjugation than sulfapyridine.

Sodium sulfadiazine by intraperitoneal injection was studied in a series comparable to those previously run on sulfapyridine and

* Supported by Grant No. 460 to Doctor Esther M. Greisheimer from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

1 Wertenberger, Grace E., *Medical Times*, 1941, **69**, 471.

2 Behrmann, V., and Fay, M., *Science*, 1939, **90**, 187.

3 Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

4 Van Dyke, H. B., Greep, R. O., Rake, G., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 410.

TABLE I.
Comparison of Effect of Oral and Intraperitoneal Administration of Sulfonamides upon Blood pH.

Compound	Dose 1 cc/100 g rat %	No. rats	Route	Observation period, hr	Avg pH			Blood levels of drug mg/100 cc blood		
					Initial	After drug	Change	Free	Total	Combined
Controls	—	10	—	3	7.51	7.50	—.01	.58	.58	0
	—	4	—	6	7.49	7.50	+.01	1.30	1.30	0
Sodium Sulfapyridine	10	13	Intraperit.	3	7.51	7.59	+.08	59.88	67.94	8.06
	10	16	Oral	3	7.48	7.48	.0	12.52	14.97	2.45
	10	11	”	6	7.47	7.50	+.03	18.79	22.14	3.35
Sodium Sulfadiazine	10	28	Intraperit.	3	7.516	7.53	+.014	—	—	—
	10	6	”	3	7.50	7.49	—.01	139.67	154.06	14.39
	10	11	Oral	3	7.50	7.50	.0	—	—	—
	10	7	”	6	7.49	7.49	.0	68.44	80.56	12.12
	1	6	Intraperit.	3	7.476	7.49	+.014	13.49	15.02	1.53
Sodium Sulfathiazole	10	14	”	3	7.51	7.58	+.07	54.63	62.49	7.86
	10	9	Oral	3	7.48	7.49	+.01	—	—	—
	10	6	”	6	7.48	7.48	.0	14.56	16.88	2.32
	1	6	Intraperit.	3	7.49	7.49	.0	4.89	6.11	1.22

sulfathiazole. It is evident that it does not produce the marked effect on the blood pH shown by the other preparations, even though the blood levels were much higher. However, there is some individual variation in the effect of the drug for 8 (about 25%) of the animals showed an increase of .04 unit or more in blood pH. The majority, however, showed no change. No evidence of toxicity was observed and no animals died during the experimental period.

It is evident that in the case of sulfapyridine and sulfathiazole single doses given intraperitoneally, if sufficiently concentrated, by their rapid absorption and sudden rise in the blood stream affect the buffer capacity of the body and give rise to an alkalosis. When the blood level rises more slowly as with oral administration and slow absorption, the buffer capacity of the body is not embarrassed and the normal blood pH is maintained.

Sulfadiazine, however, does not have this effect.

Summary. Single doses of the sodium salts of sulfapyridine and sulfathiazole cause a marked rise in the blood pH which is greatest in the case of sulfapyridine when they are given by intraperitoneal injection. This rise does not occur when they are administered orally even when the observation period is doubled and the blood level of the drug has risen somewhat higher, though it is markedly lower than the level observed three hours after intraperitoneal injection.

Sodium sulfadiazine, on the other hand, does not have this marked effect upon blood pH when administered intraperitoneally or orally even though the blood level is more than twice that observed in either of the other preparations.

I wish to express my sincere appreciation to Doctor Versa Cole for technical advice on the photoelectric colorimeter and to Doctor Esther M. Greisheimer and Doctor Roberta Hafkesbring for their assistance in this work.

13868

An Experimental Study with Tantalum.

HENRY M. CARNEY. (Introduced by J. C. Burch.)

From the Department of Surgery, Vanderbilt Medical School.

Venable, Stuck and Beach[1] introduced vitallium into surgery. This metal is an alloy of cobalt, chromium and molybdenum. While widely used and excellently tolerated by tissue, it has certain metallurgical disadvantages. It cannot be drawn into wire and cannot be worked in the cold state. This necessitates casting. For these reasons, a survey of other metals was undertaken, and on account of its properties, the element tantalum was selected. Its atomic weight is 181.4; atomic number 73. The metal is characterized by a marked resistance to chemical corrosion, being attacked only by hydrofluoric acid and concentrated alkalis. It is ductile and malleable in the cold state. Due to these properties, it seemed ideal for certain surgical procedures.

Samples were obtained from the manufacturer,* and after a few preliminary experiments it was used clinically. These early experiences were so satisfactory that its use has been continued. Publications of the results, however, have been withheld until further clinical and experimental evidence was obtained. Burke[2] reported on 34 clinical cases in which it was used as a suture material. Recently, Pudenz[3] has found the metal of great advantage in neurosurgery. Our clinical data will be reported elsewhere. The purpose of this communication is to report certain observations on the reaction of the tissues of dogs to tantalum.

Method. Tantalum plates and screws were weighed and aseptically applied to the tibia

TABLE I.

Dog No.	Site	Material	Initial wt	Duration, days	Wt at removal	Loss
2	Tibia	Tantalum Plate	6.1450	180	6.1360	.0090
	"	" Screw	1.2971	180	1.2950	.0021
	"	" "	1.3420	180	1.3400	.0050
3	"	" Plate	6.0750	150	6.0735	.0015
	"	" Screw	1.3160	150	1.3160	.0000
	"	" "	1.3100	150	1.3090	.0010
27	"	" "	1.6890	35	1.6880	.0010
	"	" "	2.0015	35	2.0013	.0002
30	"	" "	1.3394	33	1.3394	.0000
	"	" "	1.2947	33	1.2946	.0001
28	"	Iron "	.5494	32	.5430	.0064
	"	" "	.5558	32	.5505	.0053
29	"	Brass "	.5495	30	.5370	.0125
	"	" "	.5622	30	.5475	.0147
22	Abd. Wall	Tantalum Plate	6.1789	22	6.1778	.0011
23	" "	" "	6.6210	24	6.6195	.0015
24	" "	Iron "	3.5950	20	3.6024	.0074*
25	" "	Brass "	4.9290	20	4.9176	.0114
26	" "	Galv. Tin "	3.9540	20	3.9739	.0199*

*Gain.

1 Venable, C., Stuck, W., and Beach, A., *Trans. South. Surg. Assn.*, 1936, **49**, 294.

* Fansteel Metallurgical Corporation, North Chicago, Illinois.

2 Burke, G. L., *Canad. Med. Assn. J.*, 1940, **43**, 125.

3 Pudenz, R. H., unpublished data.

of dogs and were allowed to remain from 30 to 108 days. In addition, tantalum plates were buried in the abdominal wall of dogs. For comparative purposes a limited number of screws and plates of iron, galvanized tin and brass were used. At the termination of the experiment the animals were X-rayed and the metallic implants removed and weighed. The condition of the surrounding tissues was carefully noted and specimens removed for microscopic study.

X-ray Observations. The density of the bone adjacent to the tantalum plates was increased. There was also an increase in density along the shafts of the screws. In the animals with iron and brass screws there was a decrease in density and rarefaction as early as 30 to 32 days.

Weight Changes. These changes are shown in the accompanying table.

Gross Appearance of the Tissue. Surrounding the tantalum there was a thin film of shiny fibrous tissue. In no instancce was discoloration present. Beyond the film the tissues appeared normal. The periosteum of the bone adjacent to the tantalum plates was thickened. Around the iron there was a definite, but slight brownish discoloration. Brass produced a greenish discoloration and marked edema.

Microscopic Appearance of Tissue. The thin film of shiny tissue surrounding the tantalum plates was seen to be composed of fibroblasts. No edema and no inflammatory changes were noted. Sections of the decalcified bone showed no change in the bone substance and a small fibrous capsule adjacent to the periosteum. Sections of tissue exposed to other metals exhibit varying degrees of tissue reaction to the foreign body. Galvanized tin showed a large deposit on the plate, but only small amount of increase in the fibrous tissue about the plate. Brass showed an extreme tissue reaction with edema, fibrin deposit and cellular infiltration. Sections of tissue surrounding brass and iron show large amounts of metallic deposits.

Conclusions. Tantalum implanted in soft tissues is encapsulated by a thin layer of fibrous tissue. On the surface of the bone a similar reaction is observed, and in addition, X-ray reveals an increased density in the underlying bone. The metal is apparently well tolerated by tissue.

13869

A Microtome Sectioning Technique for Electron Microscopy Illustrated with Sections of Striated Muscle.

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The preparation of histological and cytological sections for study with the electron microscope presents several difficulties. These are due chiefly to the necessity of drying and to the low penetrating power of electrons.[1] The distortion commonly attendant on air-drying can be obviated by vacuum-drying in the frozen state. The low penetrating power of electrons is more serious since for high resolution electron micrographs it has been found that sections must be less than 0.25 μ and preferably less than 0.1 μ thick.[2] von Ardenne[3] has described a method, but, judging from the one micrograph published, his sections were not sufficiently thin for good resolution or adequate penetration. The present paper describes a technique by means

* RCA Fellow of the National Research Council. Present address: Johnson Foundation, University of Pennsylvania.

1 See any recent article on electron microscopy.

2 Richards, A. G., Jr., and Anderson, T. F., *J. Morph.*, 1942, **71**, in press.

3 *Z. wiss. Mikr. u. mikr. Tech.*, 1940, **57**, 291.

of which relatively uniform 0.1 μ sections of striated muscle have been prepared and examined with 60 kV electrons.

Some types of material can be cut free-hand[2] but much histological material requires a microtome. No commercial microtomes cutting such thin sections are now available but standard commercial models can be adjusted by a number of different ways to cut thinner sections. Our machinists have suggested three methods for adjusting microtomes to cut at fractions of a micron: (1) For Minot and similar sliding microtomes the cam knob that hits the setting device to control the movement of the ratchet wheel may be machined so that it has 4 quadrants each with a different radius. The radii can be adjusted so that with the machine set to cut at one micron the micrometer screw moves only approximately one-quarter of a micron (one-quarter of a tooth-spacing on the ratchet wheel) at a stroke, the cam knob being turned by hand between strokes. (2) The single micrometer screw used to move the specimen may be removed and replaced by a differential screw. (3) For indirect drive rotary microtomes, the same result may be obtained by decreasing the angle of the inclined plane. The first method is simplest but it has the least accuracy and does not give an automatic feed. In this preliminary work a Bausch and Lomb 1903 model of the Minot Automatic Precision Sliding Microtome was converted by the first method, but for further work this machine is being changed over to the more accurate differential screw drive with a small, strongly-braced specimen vise.

Muscle was fixed for 10 hours in a freshly prepared solution containing 50% ethyl alcohol, 10% formalin (=4% formaldehyde) and 5% glacial acetic acid. After washing in distilled water,† the tissue was transferred to "Carbo-Wax 4000"‡ in distilled water, the wax concentrated by evaporation of the water under a lamp, then the tissue transferred to pure molten wax (approximately 50° C) for a short while and finally blocked in a paper box.§ An infiltration time of 2 hours was used for a piece of tissue approximately 2 mm on an edge. The block was trimmed so that the surface of the specimen was at a slight angle to the surface of the wax, then placed on the specimen block of the microtome and sections cut perpendicular to the knife edge at settings on approximately 0.25 μ. Since the specimen was inclined at a slight angle, a tapering wedge of muscle was obtained in each uniformly thin slice of the wax.‖ Sections were accumulated in a dry dish. The somewhat hygroscopic wax was first softened by 15-30 minutes exposure to a saturated atmosphere, and then completely removed in distilled water.¶ Suitable sections were chosen under the light microscope and gently mounted on the wire disc[2] which serves for holding specimens in the electron microscope.** The specimens were then rapidly frozen by plunging the disc into

† Saline probably cannot be substituted here because of difficulties encountered in infiltrating with the wax. With *precipitated* protoplasm the distilled water is probably not harmful.

‡ "Carbo-wax 4000" is the trademark for a family of higher glycols prepared by The Carbide and Carbon Chemical Corporation, 30 East 42nd Street, New York City.

§ Since this manuscript was submitted, we have found that infiltration may be performed at room temperatures. Tissues are placed in a fairly deep dish of wax dissolved in water, and the water then removed in a desiccator. This necessitates 10-15 days to obtain a hard wax, but this can be accomplished without crystallization. Both heat and the shrinkage attendant on cooling the molten wax are thus eliminated.

‖ One might expect a good tapering wedge of material in any slice of an inclined specimen but in actual practice the best results are obtained from the thinnest wedges.

¶ Dropping sections directly into distilled water sets up such violent surface currents that this was discontinued in favor of a gentler method of dissolving the wax. The wax, of course, must be removed for it fills the interstices of the specimen (thereby decreasing contrast) and because it is a uniform slice containing a tapering wedge of muscle. This is one of the crucial points in the technic and the outstanding advantage of this particular wax is its ready removal with water.

** No membrane was used for support in these studies. The specimens were placed directly on clean wire screens. Specimens should lie flat on the screen and adhere to it.

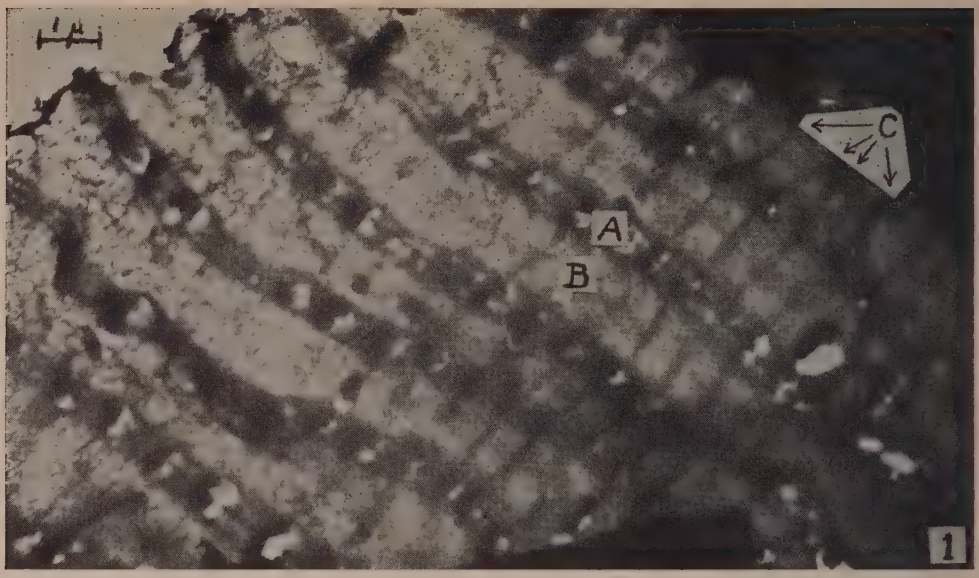

FIG. 1.

Electron micrograph of striated muscle of American coackroach. "A" is dark cross-band composed of 3 lines; "B" is faintly double cross-band in light band; "C" points to longitudinal bands. Direction of sectioning is from lower right corner to upper left corner. Original micrograph, 2200 ×, photographically enlarged to 6500 ×.

petroleum ether at the temperature of a dry-ice (solid CO_2) and alcohol mixture (—72° C). The screen was then transferred to a cold pillar (aluminum rod standing in mercury in a glass cylinder) which fitted into a vacuum system connected to a Cenco Megavac pump, and the specimens dried while frozen. Using a liquid-air trap between specimen and pump, drying was completed in 5-10 minutes. Finally, the specimens were warmed to room temperature, then exposed to air and introduced into the electron microscope.

Stereoscopic and other electron micrographs were taken of sections of striated muscle from the prothorax of the American cockroach *(Periplaneta americana)*. The sections were mostly around 0.2 μ thick but a considerable part of one section was not over 0.1 μ thick and was especially favorable for micrographing with 60 kV electrons (Fig. 1-2). The state of contraction of this muscle is not known. For cross (= transverse) striations the micrographs show dark (= denser) regions ("A") approximately 0.7 μ broad and 1.5 μ apart. Subsequent inspection of this section with the light microscope shows that these denser regions correspond to the dark cross bands seen with the light microscope. Each of these dark regions ("A" is made up of 3 equally spaced dark bands approximately 0.15 μ broad and separated by approximately the same distance. Between the composite dark bands is a lighter (= less dense) region approximately 1.5 μ broad. In the center of this light region is what appears to be a faintly double band ("B"); each of the faintly darker units of this is approximately 0.15 μ broad, 0.15 μ apart and in density intermediate between the light matrix and the dark bands mentioned first. A single muscle disc, then, shows 4 or 5 continuous cross bands; 3 dense ones closely spaced ("A") and one or 2 lighter ones ("B"). In the thinnest section and less distinctly in some of the thicker sections there are also longitudinal bands ("C") approximately 0.15 μ broad, 0.7 μ apart, and having approximately the same density as the 3 dark transverse

FIG. 2.
Photographic enlargement of a portion of Fig. 1. 23,000 ×.

bands. Stereoscopic pictures show that these longitudinal bands are really regions of greater density rather than being due to any waviness of the section (*i.e.* cutting artifacts). Their periodicity also suggests that they are true muscle components. These longitudinal bands appear to run continuously across the 5 cross bands. Note that a small hole is usually present on the right side of the longitudinal band where this band crosses the composite transverse band (the direction of knife movement was from right to left in Figures 1 and 2); this may mean that these are regions of greater hardness, at least in wax imbedded specimens. These longitudinal bands ("C") may be the myofibrillae but this cannot be stated for certain at present. Filling the interstices between all these bands and grading into them is what appears to be a gel-like material. It seems noteworthy that all the cross bands and the longitudinal bands are continuous and are of the same magnitude—approximately 0.15 μ—although not all of the same density. No further interpretation of these pictures will be attempted at this time since the object of the above description is to illustrate the results that have been obtained with this technique.

As in the usual types of histological technique slight changes in procedure will be necessary for different tissues and will have to be worked out as needed.

The slight irregularities in sharp microtome knife edges cause no appreciable difficulties in the usual histological studies (sections 5-20 μ thick) but cause relatively large linear variations in sections 0.1 μ to 0.2 μ thick. However, since only small areas are used from any one micrograph this is circumvented by selecting uniform areas for micrographing.

Other hard waxes (not soluble in water) can be used but, aside from necessitating the use of fat solvents such as alcohol and xylol, require a long hydration series with an extremely delicate section. Satisfactory results were not obtained by fixing a paraffin section onto a collodion membrane on a screen because the transferral through xylol and alcohol weakened the collodion membrane.

"Carbo-wax 4000" is satisfactory for cutting at small fractions of a micron (inclined specimens within the wax being cut as thinner wedges) and requires only water as solvent.

Full evaluation of the normality of these tissues cannot be given at present. Since fixed material is itself abnormal, more reliable results might be obtained by freeze-drying fresh tissue, sectioning and placing directly in the electron microscope. However, comparison of these micrographs with current ideas of muscle structure suggest that the results with fixed tissues will prove satisfactory.

13870 P

Exploratory Study of Early Effects of Urinary Gonadotropin in Prepuberal Male Rats.*

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Earlier experiments[1, 2] have shown that the effect of a synthetic androgen can be readily detected in the accessory reproductive organs of castrated young adult rats within about 36 hours after its administration. Since testosterone propionate is a fat soluble substance which acts directly upon the accessory reproductive organs, it is of interest to determine the effectiveness of a gonadotropic substance which acts indirectly *i.e.* through its action upon the testis.

Twenty rats at an age of 20 days (29.2 ± 1.07 g) were employed, ten of which were treated on one day only with a pregnancy urine gonadotropin (Antuitrin-S) which is considered by some as entirely luteinizing.[3] Approximately 6 hours before autopsy the rats were weighed and injected with colchicine (0.1 mg per 100 g weight of rat); the gonadotropin and the colchicine were administered subcutaneously. Mitotic activity (see table) in seminal vesicles (s.v.) and in prostate (v. pr.) is recorded as the average number per field of colchicine-arrested cells in 25 fields at a magnification of 430 diameters. The gonadotropin was administered in a single dose or in 2 doses at 8-hr intervals, each injected animal receiving a total dose of 20 rat units (25 I.U.). At selected hours after the first introduction of the hormone a treated and control animal were sacrificed in pairs; both gonadotropin treated and control animals had received the same dose of colchicine.

Results. The ventral prostate and seminal vesicles of all control rats contained mitotic cells ranging in number from 2.32 to 11.88 and 2.12 to 8.28 per field respectively. Pale light areas indicative of secretory activity are already present in the glandular epithelium of the ventral prostate;[4] mitotic cells in the seminal vesicle are fewer per field than in the prostate and the secretory cells are less differentiated. Differences in the rate of development of these two glands have been described earlier.[5]

In both groups of treated animals the

* These investigations were carried on at the University of Arkansas. Grateful acknowledgments are made to the following individuals for invaluable aid: Professors S. C. Dellinger and D. V. Holcomb, Department of Zoology, University of Arkansas, for laboratory space; Dr. S. C. Ericksen, Department of Psychology, University of Arkansas, for rats of the proper age; Miss Grace I. Zimmerman, Dept. of Anat. and Hist., University of Maryland, and Prof. T. L. Smith, Dept. of Biology, College of the Ozarks, for needed technical equipment.

1 Burkhart, E. Z., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 137.

2 Burkhart, E. Z., *J. Exp. Zool.*, 1942, **89**, 135.

3 Gustavson, R. G., and D'amour, F. E., *J. A. M. A.*, 1941, **117**, 188.

4 Moore, C. R., Price, D., and Gallagher, T. F., *Am. J. Anat.*, 1930, **45**, 71.

5 Price, D., *Am. J. Anat.*, 1936, **60**, 79.

TABLE I.
Mitotic Activity in the Accessory Sex Glands of 20-day-old Rats Treated with Antuitrin-S.
(All the animals received colchicine.)

Time in hrs after 1st inj.	Group I				Group II			
	Control		20 R.U. Antuitrin-S in 2 doses		Control		20 R.U. Antuitrin-S in 1 dose	
	v.pr.	s.v.	v.pr.	s.v.	v.pr.	s.v.	v.pr.	s.v.
13					5.64	2.84	2.64	5.04
20	7.36	8.0	6.6	7.76				
23					3.72	8.28	4.8	8.88
28					11.52	7.36	4.64	27.64
28					—	3.32	0.56	26.56
30	3.16	2.12	2.96	63.64				
34					2.32	2.88	12.2	75.92
35	10.08	6.24	16.68	68.80				
43	11.88	5.64	37.2	69.16				
44					4.88	3.44	28.48	68.04

effect of gonadotropin is apparent by the sudden rise in the number of mitotic cells in the seminal vesicle at 28 and 30 hours. Increased mitotic activity lags in the ventral prostate; only slight increases occur at 34 and 35 hours but by 43 to 44 hours the increase becomes significant.

These limited data show clearly that a single 20 R.U. dose of urinary gonadotropin is effective and detectable. It is interesting to note that despite the indirect action of gonadotropin, via the testis, the effect of the Antuitrin-S is elicited several hours earlier in prepuberal rats than is testosterone injected into castrated young adults.[2]

Conclusions. The effect of a urinary gonadotropin is apparent in the accessory organs of reproduction of 20-day-old intact male rats by an increase in mitotic activity, arrested by colchicine; seminal vesicle response occurs earlier than prostate response —28-30 hours as compared with 35-43 hours. A single 20 R.U. dose is as effective as 2 equally divided doses.

13871

Effect of Benzophenone and Allied Compounds on Human Tubercle Bacilli *in vitro*.

B. L. FREEDLANDER. (Introduced by Ernest L. Walker.)

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1. *Bacteriostatic Action on a Rapidly Growing Avirulent Strain.* The bacteriostatic action of benzophenone (diphenyl ketone) and its derivatives was tested on an avirulent Novy strain of tubercle bacilli. This culture normally produces a heavy surface growth within 4 days. A liquid synthetic asparagin media, containing 6.% glycerine, was used, with pH 7.2. The chemical compounds, with a few exceptions, were dissolved in propylene glycol to make 1.% solutions. Subsequent dilutions were made directly into the media. Twenty-seven compounds, listed in Table I, were tested.* The amino derivatives were

* Most of the chemicals were obtained from the Eastman Kodak Co., Rochester, N.Y. Those which were impure were recrystallized. o-Chlorobenzophenone was synthesized by the Friedel and Crafts reaction with o-chlorobenzoyl chloride, benzene, and aluminum chloride. Promin was obtained from Parke, Davis & Co., Detroit, Mich.

Table I

Bacteriostatic Action on Growth of Avirulent Tubercle Bacilli *in vitro*

	Compound		Highest Bacteriostatic Dilution
1.	Benzophenone		1:10,000
2.	p-Hydroxybenzophenone	$C_6H_5 \cdot CO \cdot C_6H_4OH$	1:5,000
3.	p Methoxybenzophenone	$C_6H_5 \cdot CO \cdot C_6H_4 \cdot OCH_3$	1:20,000
4.	4,4' Dimethoxybenzophenone	$CH_3O \cdot C_6H_4 \cdot CO \cdot C_6H_4 \cdot OCH_3$	< 1:3,000
5.	p-Aminobenzophenone	$C_6H_5 \cdot CO \cdot C_6H_4NH_2$	1:5,000
6.	4,4' Diaminobenzophenone	$NH_2 \cdot C_6H_4 \cdot CO \cdot C_6H_4NH_2$	< 1:3,000
7.	Tetramethyldiaminobenzophenone	$(CH_3)_2NC_6H_4 \cdot CO \cdot C_6H_4N(CH_3)_2$	< 1:3,000
8.	Tetraethyldiaminobenzophenone	$(C_2H_5)_2NC_6H_4 \cdot CO \cdot C_6H_4N(C_2H_5)_2$	< 1:3,000
9.	p-Chlorobenzophenone	$C_6H_5 \cdot CO \cdot C_6H_4Cl$	1:40,000
10.	o-Chlorobenzophenone	$C_6H_5 \cdot CO \cdot C_6H_4Cl$	1:40,000
11.	2,4' Dichlorobenzophenone	$Cl\,C_6H_4 \cdot CO \cdot C_6H_4Cl$	1:100,000
12	4,4' Dichlorobenzophenone	$Cl\,C_6H_4 \cdot CO \cdot C_6H_4Cl$	< 1:3,000
13.	Phenyl-p-tolyl Ketone	$C_6H_5 \cdot CO \cdot C_6H_4CH_3$	1:20,000
14.	Di-p-tolyl Ketone	$CH_3C_6H_4 \cdot CO \cdot C_6H_4 \cdot CH_3$	< 1:3,000
15.	o Benzoylbenzoic Acid	$C_6H_5 \cdot CO \cdot C_6H_4 \cdot COOH$	< 1:3,000
16.	Diphenyl Sulfone	$C_6H_5 \cdot SO_2 \cdot C_6H_5$	< 1:3,000
17	Di-p-tolyl Sulfone	$CH_3 \cdot C_6H_4 \cdot SO_2 \cdot C_6H_4 \cdot CH_3$	< 1:3,000
18.	Promin	$(C_6H_4)_2 \cdot SO_2\left[NH \cdot CH \cdot (CHOH)_4\,CH_2OH \cdot SO_3NA\right]_2$	< 1:3,000
19.	Diphenyl Sulfoxide	$C_6H_5 \cdot SO \cdot C_6H_5$	1:5,000
20.	Benzhydrol	$C_6H_5\,CHOH \cdot C_6H_5$	1:5,000
21.	Tetramethyldiaminobenzhydrol	$(CH_3)_2\,N \cdot C_6H_4 \cdot CHOH \cdot C_6H_4 \cdot N(CH_3)_2$	1:5,000
22.	Benzanilide	$C_6H_5 \cdot CO \cdot NH \cdot C_6H_5$	< 1:3,000
23.	Benzil	$C_6H_5 \cdot CO \cdot CO \cdot C_6H_5$	< 1:3,000
24.	o-Chlorobenzoic Acid	$ClC_6H_4 \cdot COOH$	< 1:3,000
25.	p-Chlorobenzoic Acid	$ClC_6H_4 \cdot COOH$	1:3,000
26.	o-Chlorobenzoyl Chloride	$ClC_6H_4 \cdot COCl$	< 1:3,000
27.	p-Chlorobenzoyl Chloride	$ClC_6H_4 \cdot COCl$	< 1:3,000

dissolved in dilute HCl, as well as in propylene glycol. Promin was dissolved in water. Controls were run with all diluents. As many of the compounds formed varying amounts of precipitates in the aqueous media, the bacteriostatic actions are not strictly comparable. The results (Table I) show only the highest bacteriostatic dilution for each compound: this was taken as that dilution which showed no macroscopic growth within 6 days. The compounds are listed with their formulae in an order which illustrates the relation between chemical structure and inhibitory action.

The parent compound, benzophenone, showed a moderately high inhibitory effect in a concentration of 1:10,000. p-Hydroxybenzophenone inhibited to a lesser degree (1:5,000). p-Methoxybenzophenone gave a high bacteriostatic effect in 1:20,000 concentration: however, the di-p-methoxy derivative showed a decidedly decreased inhibitory effect, (less than 1:3,000). The p-amino derivative (no. 5) prevented growth only in 1:5,000 dilution. 4,4′-Diaminobenzophenone, as well as its tetramethyl and tetraethyl derivatives, were low in bacteriostatic effect (less than 1:3,000).

The chlorine derivatives showed the highest degree of inhibition of the compounds tested. Both the ortho and the para chlorobenzophenone inhibited growth in a dilution of 1:40,000, while the 2,4′-dichlorobenzophenone inhibited in a concentration of 1:100,000. The 4.4′-dichlorobenzophenone, on the other hand, prevented growth only in a dilution of less than 1:3,000. All the chlorine derivatives gave heavy precipitates in the media in the lower dilutions. Two alkyl derivatives (nos. 13 & 14) were tested. Phenyl-p-tolyl ketone showed a good inhibition, in concentration of 1:20,000; the dimethyl derivative (di-p-tolyl ketone) was bacteriostatic in dilution of less than 1:3,000. o-Benzoyl benzoic acid was the only carboxyl derivative tested: this did not inhibit in dilution of 1:3,000.

Benzhydrol and its tetramethyldiamino derivative (nos. 20 & 21), wherein the central ketone group is partially reduced, were bacteriostatic in concentration of 1:5,000. Benzanilide and the diketone, benzil, showed poor inhibition. Diphenyl sulfoxide prevented growth in dilution of 1:5,000. The diphenyl sulfone was slightly inferior to the sulfoxide, while di-p-tolyl sulfone and promin were no better. Both ortho and para chlorobenzoic acids were poor, as were also ortho and para chlorobenzoyl chlorides.

2. *Bacteriostatic Action of 2,4′-Dichlorobenzophenone on a Slow Growing Virulent Strain. A. In Liquid Media.* A modified Sauton's media was used, 100 cc to each flask. Six flasks were used for each dilution. The strain of tubercle bacilli employed was taken from the first transplant of a growth isolated from sputum. A heavy suspension was made in buffered saline (pH 7.0); 0.2 cc of this suspension, which contained approximately 20 bacilli per oil immersion field, was inoculated into each flask. The drug was tested in concentrations of 1:10,000, 1:30,000, 1:60,000. Controls were run with 1.% propylene glycol. Readings were made at the end of six weeks. The compound inhibited growth in all flasks, including the 1:60,000 dilution, which was the highest dilution tested. Growth in the controls was heavy.

B. In Solid Media. Growth was tested on Lowenstein's egg media. Dilutions of the drug were made in asparagin solution, the egg was then added and the mixture sterilized by heat. No bacteriostatic action was noted. Apparently sterilization of the compound in media with heat destroys its effect.

Summary and Discussion. Various derivatives of benzophenone inhibited the growth of tubercle bacilli. 2,4′-Dichlorobenzophenone was bacteriostatic in high dilution, ortho and para chlorobenzophenone to a lesser degree. These compounds were effective in spite of their relative insolubility in the aqueous media. Attempts are being made to increase the solubility of these compounds without decreasing their bacteriostatic effect. Other halogen derivatives are also being investigated. The para methyl and methoxy derivatives also inhibit growth. It is possible that other higher alkyl derivatives may show an increased effect. All of the di-para de-

rivatives (amino, methyl, methoxy, chloro) were inferior to the mono para compounds. Substitution of the hydrogen atoms of the amino groups with alkyl groups did not increase the effect. Benzil, benzanilide, and o-benzoyl benzoic acid did not inhibit in the lowest dilution. The sulfoxide and sulfone derivatives, including promin, were low in bacteriostatic action, although no analogous halogen or alkyl derivatives of these compounds were tested. 2,4′-Dichlorobenzophenone also inhibited a virulent strain of tubercle bacilli in high dilution in liquid media, but not on solid media.

13872

Lactogenic Hormone Prolongs the Time During Which Deciduomata May be Induced in Lactating Rats.*

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The ability to produce deciduomata is an indicator of corpus luteum activity. This functional test has been used as evidence that the corpora were active during lactation in the rat. Inasmuch as deciduomata can be produced as late as the sixteenth day of lactation,[1] the corpora function normally for approximately this duration. Although the corpora do not function for any appreciable period after this time, it has been possible by progesterone administration to prolong the period during which deciduomata may be produced.[2]

Recently[3] purified lactogenic hormone has been given hypophysectomized rats to produce deciduomata. This appears to demonstrate a gonadotropic function of this hormone. In these experiments the corpora lutea appeared large and healthy even when the follicles, as well as the interstitial tissue were below normal. Furthermore in this same work the effectiveness of lactogenic hormone in producing the growths in adrenalectomized rats was demonstrated.

Fifteen lactating rats were subjected to uterine stimulation as late as the twenty-fourth postpartum day and were thereafter injected subcutaneously with 100 I.U. of com-

TABLE I.

Prolongation of Luteal Function by Lactogenic Hormone.

Uterine stimulation postpartum day	Test rats	Deciduoma	Control rats	Deciduoma
20	3	3	2	0
21	3	3	2	0
22	3	3	2	0
23	4	3	2	0
24	2	1	—	—
Total	15	13	8	0

* This study was aided by the Christine Breon Fund for Medical Research.

1 Lyon, R. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 151.

2 Unpublished experiments.

3 Evans, H. M., Simpson, M. E., Lyons, W. R., and Turpeinen, K., *Endocrinology*, 1941, **28**, 933.

mercial lactogenic hormone† daily for 4 days. As shown in the table, 13 of the 15 animals, sacrificed on the 5th day, showed microscopically confirmed deciduomata, together with large robust-appearing corpora lutea. Eight uninjected control animals showed small regressive corpora and no deciduomata at the sites previously stimulated. The results are interpreted as further evidence of corpus luteum stimulation by the lactogenic hormone.

Presumably this gonadotropic function is mediated by the continued production of progestin in the corpora in sufficient concentration to permit the growth of the deciduomata well past the time when they can normally be produced.

† Acknowledgment is made of the donation of commercial lactogenic hormone of the anterior pituitary gland to Schering Corporation, Bloomfield, N.J.

13873

Rôle of Inositol and p-Aminobenzoic Acid in Normal Lactation.

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The fact that several members of the vitamin B complex are required for lactation in the albino rat has long been recognized. Almost every member of the complex has at one time or another been suggested for a crucial role. The list includes thiamin,[1] riboflavin,[2] pantothenic acid,[3] choline,[4] p-aminobenzoic acid,[5] and inositol.[5] Less well defined factors which have been reported as essential are factor W[6], and the filtrate factor.[7] In most cases these reports lack confirmation from other workers.

The pioneer work of Sure[5] indicated that p-aminobenzoic acid is essential for lactation in the albino rat and that inositol may be.

The conclusions drawn from the work were evidently not intended to be final, and it has seemed worthwhile to examine critically the role played by each of these substances.

Methods. Young female rats from our own breeding colony were divided into groups of 12 and placed on the following dietary regimes shortly after weaning:

Group A. Normal breeding diet* containing all known dietary elements. We have found over a period of 5 years that this diet is capable of meeting all the requirements of breeding rats.

Group B. Deficient diet† plus B-complex supplement.‡

	%
* Corn	28.0
Wheat	28.0
Whole milk powder	22.5
Linseed meal	8.0
Alfalfa	3.0
Crude casein	4.0
Calcium carbonate	0.5
Sodium chloride	0.5
Brewer's yeast	3.0
Cod liver oil	2.5
	g
† Casein[6]	25.0
Agar agar	2.0
Cystine	0.2
Butter fat	10.0
Dextrin	58.8
Salt mixture 351	4.0
Cod liver oil	2.5
Mixed toxopherols	0.25
2-methyl,1,4-naphthoquinone	0.01

(This diet was made up at 5-day intervals and kept under refrigeration until used.)

‡ Thiamin[5]	120 γ
Riboflavin	120 γ
Pyridoxine	120 γ
Calcium pantothenate	600 γ
Nicotinic acid	6 mg
Choline hydrochloride	15 mg

(The amounts indicated were the average daily supplement per rat.)

1 Evans, H. M., and Burr, G. O., *J. Biol. Chem.*, 1928, **76**, 263.

2 Hussemann, D., and Hetler, R. A., *J. Nutrition*, 1931, **4**, 127.

3 Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 625.

4 Sure, B., *J. Nutrition*, 1940, **19**, 71.

5 Sure, B., *J. Nutrition*, 1941, **22**, 499.

6 Sure, B., *J. Nutrition*, 1940, **19**, 57.

7 Morgan, A. F., and Simms, H. D., *Science*, 1939, **89**, 565.

FIG. 1.

Mortality rates of newborn, average daily milk secretion for mothers, and average daily weight increments of pups on various vitamin B-complex regimes (see text).

Group C. Deficient diet plus B-complex supplement plus p-aminobenzoic acid (15 mg per day).

Group D. Deficient diet plus B-complex supplement plus inositol (15 mg per day).

Group E. Deficient diet plus B-complex supplement plus p-aminobenzoic acid and inositol (each 15 mg per day).

All rations were supplied *ad libitum* and the various supplements were administered by stomach tube. Supplements were administered daily except Sundays but this deficiency was overcome by giving 50% greater doses on Saturdays and Mondays.

The B-complex supplement was so prepared that it was contained in 0.5 cc of water. The p-aminobenzoic acid and inositol supplements were so prepared that 0.25 cc of the aqueous solution contained 15 mg. (The former was present as the sodium salt.)

Weights were followed carefully for each animal. When they reached a weight of about 180 g they were placed for a period of 10 days in groups of 3 with an adult male of proven fertility. At the end of this time the males were returned to stock and the experimental animals were placed in individual cages. Litter weights were obtained daily from the date of birth.

Results. The experimental animals all grew at satisfactory rates. However, it was noticeable that group A grew somewhat better than any of the animals receiving the deficient diet. This phenomenon strongly resembles that observed by Troescher-Elam and Evans[8] in mice, and the growth curves in general closely resembled those presented by Sure.[5] Fig. 1 shows the average daily milk secretion for the mothers, as indicated by the total weight increment of the litter, and the average daily weight increments for the pups. The cumulative infant mortality rates, as percentages of the total births, appear at the top of the figure.

Conclusions. The results indicate that the B-complex supplement without p-aminobenzoic acid or inositol will not support normal lactation, that under these conditions the initiation of lactation is slow, and that it never achieves a normal level. The rapid decline in milk production beginning with the tenth postpartum day is also noteworthy; it culminates in almost complete cessation of lactation on the 14th day.

The addition of p-aminobenzoic acid to the B-complex supplement appears to result in

[8] Troescher-Elam, E., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 549.

delayed initiation of lactation, there being no significant amount of milk until the 5th postpartum day. After this time, however, secretion was maintained at a normal level.

With the addition of inositol to the B-complex supplement there was a prompt initiation of lactation which was maintained at a level only slightly less than that of the controls. Further supplementation of this regime with p-aminobenzoic acid resulted in no significant improvement in milk secretion over that in the group which received the B-complex supplement and inositol alone.

These findings are in general supported by the infant mortality rates for the various experimental groups. From these data we may conclude that the B-complex supplement alone or with the addition of p-aminobenzoic acid will not support normal nutrition of young rats. Supplementation with inositol results in mortality rates only slightly greater than those observed in the control animals. Supplementation with both inositol and p-aminobenzoic acid results in mortality rates quite comparable to those of the controls.

Summary. The critical role of inositol in the maintenance of normal lactation in the albino rat has been confirmed. P-aminobenzoic acid does not seem to increase lactation directly but when given to animals also receiving inositol it slightly decreases mortality rates of the newborn.

13874

Action of Various Steroids on the Hypophysis of the Thyroidectomized Rat.

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The typical modification in the hypophysis of the thyroidectomized rat, namely the degranulation of the eosinophiles and the appearance of large basophilic vacuolated "thyroidectomy cells" are known to be prevented by treatment with thyroid extract,[1-3] but the effect of steroid hormones on the development of thyroidectomy changes has not been definitely established as yet. Zeckwer[2] reported that estrone does not prevent the occurrence of thyroidectomy cells, while Nelson and Hickman[4] claimed that it completely inhibits their development. Severinghaus[3] concluded—partly on the basis of similar experiments—that there is: "sufficient evidence not only to identify these thyroidectomy cells as basophiles, but also as modified basophiles similar in characteristics to castration cells"; it was pointed out, however, that the modifications of the acidophiles are not affected by folliculoid compounds. Subsequently Nelson[5] stated that the prevention of thyroidectomy cells requires three times more estradiol benzoate than the inhibition of castration cell formation.

More recently Clarke *et al.*[6] found that all hormonally active steroids (including folliculoids, testoids, luteoids and corticoids) inhibit the development of castration cells to some extent. In view of these observations it appeared of interest to establish whether the ability to prevent the formation of thyroidectomy cells is also a morphogenetic action common to all steroid hormones.

Table I summarizes our relevant experiments. It will be noted that male albino rats weighing 130-150 g were used throughout. These were thyroidectomized the day before the initiation of treatment and killed 22 days later. Each group consisted of 6 rats. All

1 Hohlweg, W., und Junkmann, K., *Arch. f. d. ges. Physiol.*, 1933, **232**, 148.

2 Zeckwer, I. T., *Am. J. Path.*, 1938, **14**, 773.

3 Severinghaus, A. E., *Sex and Internal Secretion*, Baltimore, 1939, 3rd Ed., p. 1045.

4 Nelson, W. O., and Hickman, J., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 828.

5 Nelson, W. O., *Federation Proc.*, 1942, **1**, 63.

6 Clarke, E., Albert, S., and Selye, H., *Anat. Record*, 1942, **83**, 449.

TABLE I.

No. of Group	Treatment	M.P. °C	Dosage in mg/day	Pituitary in mg	Thyroidectomy cells
1	*Peanut oil* (0.1 cc twice daily)	—	—	8.2 (7.3-8.9)	+
2	$\Delta^{1,3,5}$-estratriene-3,17(α)-diol *α-estradiol*	176 (176-177)	.05	18 (13-22)	0
3	17-ethinyl-$\Delta^{1,3,5}$-estratriene-3,17(α)-diol *Ethinyl estradiol*	138-139 (145-146)	.05	15 (15-16)	0
4	Δ^4-androstene-3-one-17(α)-ol *Testosterone*	154 (154-154.5)	10	7.8 (7.3-8.5)	+
5	Δ^5-androstene-3(β),17(α)-diol *Androstenediol*	182-183 (182-183)	10	8.3 (7.5-10.0)	+
6	Δ^5-androstene-3(β)-ol-17-one *Dehydro-iso-androsterone*	137 (144-146)	10	8.2 (6.5-9.4)	+
7	17-ethyl-etiocholane-3(α),20(α)-diol *Pregnanediol*	237 (237-239)	10	7.3 (4.1-9.0)	+
8	17-ethyl-etiocholane-3,20-dione *Pregnanedione*	118 (120)	10	7.6 (5.4-9.3)	+
9	17-ethyl-Δ^4-androstene-3,20-dione *Progesterone*	128 (128)	10	7.1 (5.1-9.0)	+
10	17-ethyl-Δ^4-androstene-3,20-dione-21-ol acetate *Desoxycorticosterone acetate*	152 (158-160)	10	7.5 (5.9-8.3)	+
11	17-ethyl-Δ^5-androstene-3(β)-ol-20-one *Pregnenolone*	186 (185-187)	10	6.9 (4.8-8.1)	+
12	17-ethinyl-Δ^4-androstene-3-one-17-ol *Ethinyl testosterone*	265-268 (270-272)	10	8.6 (7.9-9.3)	+

animals were injected twice daily, the controls with 0.1 cc of peanut oil and the other groups with various steroids administered as a fine crystalline suspension in the same amount of peanut oil. In addition to the common name (in italics), the descriptive systematic chemical name of each steroid is given as well as the melting point of our sample. This helps to avoid any doubt concerning the nature of the material used. The melting points of especially highly purified preparations as described in the literature, are mentioned in brackets underneath the melting point of our sample in order to facilitate the estimation of the latter's purity. The average pituitary weights (with the range in brackets) are recorded as well as the presence or absence of thyroidectomy cells.

Perusal of the table indicates that although 50 γ of estradiol or ethinyl estradiol sufficed to prevent the formation of thyroidectomy cells, none of the other steroids share this effect in doses of 10 mg per day. It is quite possible that even smaller doses of the 2 folliculoid substances would have sufficed to prevent the formation of castration cells, while still larger doses of the other steroids may have proved effective. It must be kept in mind, however, that a dose of 10 mg per day is a huge amount for a rat and that this—and even smaller doses—proved highly effective in counteracting the formation of castration cells in the gonadectomized male or female rats examined by Clarke *et al.*[6] It will be noted that the folliculoids caused a pronounced enlargement of the pituitary and upon histological examination the anterior lobe cells proved greatly enlarged and almost completely devoid of chromophil granules. Indeed, the action of thyroidectomy and the folliculoids appears to be additive in this respect since the loss of eosinophil granules was even greater in thyroidectomized than in intact male rats treated with the same amount of estradiol. Thus the loss of eosinophile granules, which is normally caused by thyroidectomy, was not prevented and it remains doubtful whether the absence of vacuolized thyroidectomy cells in groups 2 and 3 should be ascribed to a "cure" of the thyroidectomy changes by the folliculoid compounds such as is obtained, for instance, with thyroid extract. These steroids, which in the intact rat cause enlargement of anterior lobe cells and degranulation of the eosinophiles, appear to have exhibited this effect even in our thyroidec-

tomized animals and it is possible that the absence of thyroidectomy cells in the latter is merely due to the fact that the usual morphogenetic effect of folliculoid overdosage prevails over that of thyroidectomy.

Since all hormonally active steroids possess some degree of folliculoid potency[7] it may be surprising to note that they do not share the anti-thyroidectomy cell effect of estradiol. This is not unexpected, however, if we consider that the action of estradiol on the size and structure of the normal hypophysis is partly inhibited by simultaneous treatment with steroid hormones having testoid, luteoid or corticoid activity.[8] In view of this observation it appears understandable that the slight folliculoid activity of the hormonally active androstane and ethyl-androstane derivatives is masked and cannot evidence itself as far as the hypophysis is concerned. That is, such compounds cause no significant enlargement of the anterior lobe cells nor degranulation of the eosinophiles. It is quite possible that in the case of castration changes the various hormonally active steroids effect a physiological substitution therapy by virtue of their folliculoid activity even if the latter cannot manifest itself by morphological signs of overdosage (enlargement in size, degranulation of eosinophiles), while in the case of the thyroidectomy cells the folliculoids act merely by virtue of their pharmacological overdosage effects so that the formation of these cells cannot be inhibited unless overdosage changes are produced. It must be admitted, however, that if it were technically feasible to give even larger doses of the above androstane and ethyl-androstane derivatives, the thyroidectomy changes might be prevented by them since Nelson[5] showed that larger doses of folliculoids are required to achieve this latter result than to prevent castration changes.

Summary. Experiments on thyroidectomized rats indicate that the formation of vacuolized "thyroidectomy cells" in the pituitary is prevented by daily doses of 50 γ of estradiol or ethinyl estradiol but remains unmodified by as much as 10 mg a day of testosterone, androstenediol, dehydro-*iso*-androsterone, pregnanediol, pregnanedione, progesterone, desoxycorticosterone acetate, pregnenolone or ethinyl testosterone. Unlike the formation of "castration cells" the development of "thyroidectomy cells" is hence not inhibitable by all hormonally active steroids even if they are administered in these comparatively high dosages.

The cost of this investigation has been defrayed by the Blanche E. Hutchinson Fund of McGill University. The authors are greatly indebted to Drs. G. Stragnell and E. Schwenk of the Schering Corporation of Bloomfield, N.J., for supplying compounds 2, 3, 4, 5, 6, 9, 10 and 12, and to Dr. S. Cook of Ayerst, McKenna and Harrison of Montreal for compounds 7 and 8.

7 Selye, Hans, *Rev. Canad. de Biol.*, 1942, **6**, 577.

8 Selye, Hans, *Canad. Med. Assn. J.*, 1940, **42**, 113.

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Oxidation of Steroids by *Alcaligenes faecalis.*

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Previous studies[1, 2] have shown that a strain of *Alcaligenes faecalis,* isolated from the cecum of a guinea pig, oxidizes cholic acid (3,7,12-trihydroxycholanic acid) to ketocholanic acids—the end product being 3,7,12-triketocholanic acid. It seemed of interest to determine whether this organism could also oxidize other bile acids and such steroids as estradiol, estriol and dehydroisoandrosterone.

Experimental. Methods. Duplicate quantities of desoxycholic, hyodesoxycholic and lithocholic acids and estradiol, estriol and dehydroisoandrosterone* were placed in 250 cc Erlenmeyer flasks and sterilized by autoclaving, after which 10 cc of sterile sheep serum were added to each flask. One cc of a heavy suspension of *Alcaligenes faecalis*† in infusion broth was added to one of each pair of flasks; 1 cc of sterile broth was added to the other flask, which served as a control. All flasks were incubated at 37.5° for 6 days and were shaken 3 to 5 times daily throughout this period. At the end of incubation, the contents of each flask were extracted with 200 cc ethyl alcohol and the extracts analyzed for ketosteroids according to a modification‡ of the Hughes procedure.[3, 4]

Results. Keto-derivatives were formed when *Alcaligenes faecalis* was cultured in serum containing desoxycholic, hyodesoxycholic and lithocholic acids and dehydroisoandrosterone (Table I). Under the same experimental conditions no keto-derivatives were formed from estradiol and estriol.

The weights of mercuric iodide hydrazones derived from digests of desoxycholic acid and dehydroisoandrosterone correspond closely to the theoretical weights required for conversion of the hydroxyl groups of these compounds to carbonyl groups. Since previous experiments[2] showed that *Alcaligenes faecalis* could oxidize hydroxyl groups at C_3 and C_{12} to carbonyl groups, it seemed probable that this organism converted desoxycholic acid to 3,12-diketocholanic acid and dehydroisoandrosterone to androstenedione. Bacterial oxidation of dehydroisoandrosterone to androstenedione was reported previously by Mamoli and coworkers,[5] who found that an organism similar to *Corynebacterium helvolum* accomplished this conversion.

The weights of mercuric iodide hydrazones obtained from digests of hyodesoxycholic and lithocholic acids were considerably smaller than those theoretically required for oxidation of the hydroxyl groups of these compounds to carbonyl groups. Incomplete conversion of lithocholic acid was probably due in part to the insolubility of this compound in serum. Incomplete conversion of hyodesoxycholic acid may have been due to the limitation of the action of *Alcaligenes faecalis* to the C_3 hydroxyl—the weight of mercuric

1 Schmidt, L. H., and Hughes, H. B., *J. Biol. Chem.,* 1942, **143**, 771.

2 Schmidt, L. H., Hughes, H. B., Green, M. H., and Cooper, E., *J. Biol. Chem.,* 1942, **145**, 229.

* We are indebted to Dr. Willard M. Hoehn, George A. Breon Co., Kansas City, Mo., for desoxycholic, hyodesoxycholic and lithocholic acids, and to Dr. Erwin Schwenk, Schering Corp., Bloomfield, N.J., for dehydroisoandrosterone, estradiol and estriol.

† The strain of *Alcaligenes fæcalis* used in this study was isolated from a guinea pig cecum in November, 1940, and has been passed through infusion broth daily since that time. The organism suspension was obtained from agar slant cultures that had been incubated for 18 hours, the organisms from each slant being washed off with 2 cc of infusion broth.

‡ The original Hughes procedure[3] was modified to eliminate the lipids contained in serum extracts. These lipids were removed by ether extraction of the lipid-hydrazone mixtures at pH 7.

3 Hughes, H. B., *J. Biol. Chem.,* 1941, **140**, 21.

4 Hughes, H. B., *J. Biol. Chem.,* 1942, **143**, 11.

5 Mamoli, L., Koch, R., and Teschen, H., *Naturwissenschaften,* 1939, **27**, 319.

TABLE I.
Oxidation of Steroids by *Alcaligenes fæcalis.*

Steroid tested	Amt steroid added to 10 cc serum, mg	Reaction mixture	Mercuric iodide hydrazone*	
			Derived from digest after incubation, mg	Theoretical for conversion of hydroxyl to carbonyl groups, mg
Desoxycholic (3,12-dihydroxycholanic) acid	20	Inoculated	79.8	82.2
	20	Control	0	
Hyodesoxycholic (3,6-dihydroxycholanic) acid	20	Inoculated	41.6	82.2
	20	Control	0	
Lithocholic (3-hydroxycholanic) acid	20	Inoculated	14.6	52.2
	20	Control	0	
Dehydroisoandrosterone	16	Inoculated	78.8	77.4
	16	Control	47.5	

*The theoretical weights of hydrazone were calculated according to equations described previously.[3] The conversion factors for the various hydroxysteroids are: 4.110 for desoxycholic and hyodesoxycholic acids; 2.599 for lithocholic acid; 4.836 for dehydroisoandrosterone.

iodide hydrazone obtained being approximately 90% of that required for conversion of just one hydroxyl to a carbonyl group.

Summary. Alcaligenes faecalis, cultured in serum, oxidized desoxycholic acid, hyodesoxycholic acid, lithocholic acid and dehydroisoandrosterone to keto-derivatives but had no action on estradiol and estriol.

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Electrophoretic Studies on New-Born Calf Serum.

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The composition of new-born calf serum before the ingestion of colostrum is completely different from that of the adult, and different as well from that of the calf after a short period of nursing. The very rapid changes in serum composition that take place during nursing were first studied by Howe[1] by salting-out methods. He found that the blood of a new-born calf "does not contain euglobulin nor pseudoglobulin I," and that after ingestion of colostrum it contains "relatively large" amounts of these globulins. We have confirmed and extended his observations by the electrophoretic study of the serum of new-born, nursing, and mature calves. Phase-rule studies have also been carried out by one of the authors.[2]

Fig. 1 shows schlierren pictures of the proteins separated by electrophoresis in new-born calf serum, and serum from 18-hour, 3-day, 5-day, and 2-year-old calves. The great difference in composition is quite obvious, both the number of fractions present and the concentration of the protein fractions change during nursing. Using the terminology first proposed by Tiselius and now commonly used in electrophoretic work, we can say that the γ fraction is absent in the new-born serum, appears when colostrum is first ingested, increases very rapidly during early nursing, and

* Now at the University of Santa Clara.

1 Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 115.

2 Jameson, E., and Roberts, D. B., *J. Gen. Physiol.*, 1937, **21**, 249.

TABLE I.
Changes in Protein Composition of Serum of Calves at Different Ages. Per cent of the Total Serum Protein Corresponding to Each Electrophoretic Fraction.

Age	γ	β	α	Albumin
New-born	—	5.9	36.8	57.3
18 hrs	6.0	9.2	35.3	49.5
36 "	41.6	7.2	21.8	29.8
3 days	49.2	5.7	17.8	27.3
5 "	48.7	6.0	10.5	34.9
2 years	43.7	6.2	9.9	40.2

TABLE II.
Mobilities at pH 7.7 and 0.5°C Corresponding to Calf Serum Protein Fractions Shown in Fig. 1.

Age	γ	β	α	Albumin
New-born	—	—	4.66	6.53
18 hrs	—	3.49	5.43	7.38
36 "	2.11	3.80	5.79	7.58
3 days	2.30	3.69	5.17	6.85
5 "	1.86	3.26	4.55	6.28
2 years	1.74	3.43	4.65	6.55

$\mu \times 10^5$ cm^2 sec^{-1} volt^{-1}.

finally decreases as the animal matures. This fraction, then, follows the same course as Howe's euglobulin.

The β fraction is present in rather low concentrations at birth, and increases slowly apparently passing through a maximum concentration as nursing progresses. The α fraction is found in extremely high concentration at birth, and its concentration decreases through nursing period reaching its lowest value in the mature serum. The albumin concentration is higher than normal at birth, decreases rapidly during nursing and then increases slightly as the animal matures. The data in Table I show the percent of the total protein that corresponds to each of these fractions for the different sera studied.

Our findings, then, roughly parallel those of Howe. Since there does not appear to be a definite correlation between protein fractions obtained by salting out and those separating by electrophoresis, it is impossible to try to compare these results more closely.

A study of the mobility data (Table II), discloses a second interesting phenomenon. During the period of greatest change in composition, that is during early nursing, the serum contains protein of markedly high mobility, so that it is questionable to assume that these proteins are the same as those present

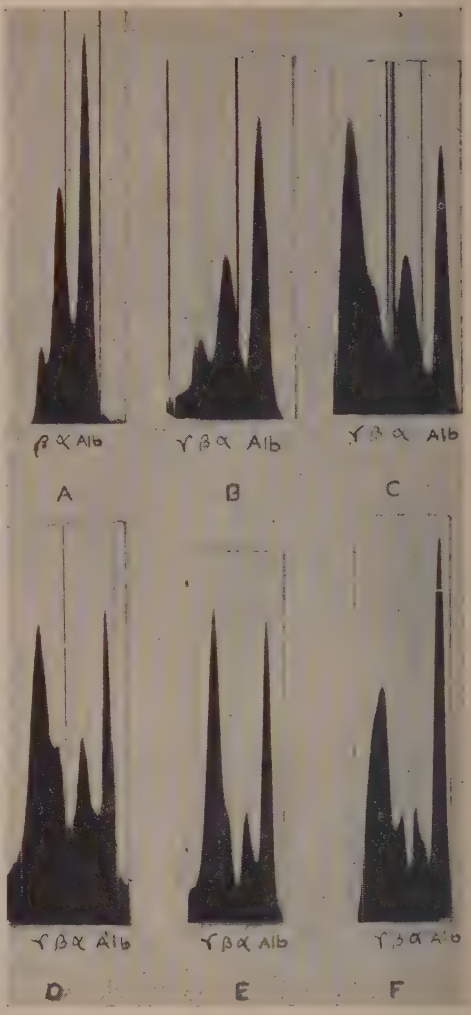

FIG. 1.
Developing Proteins in New-born Calf Serum.
A—New-born calf serum
B—18-hr-old " "
C—36 " " " "
D—3-day " " "
E—5 " " " "
F—2-yr " " "
Buffer pH 7.7. .02 M phosphate. 0.15 M NaCl. Voltage = 2 or 2.5 volts/cm. Temperature = 0.5°C. Black lines are to aid in measurements of mobilities.

in normal adult serum. They are called albumin, α, β, and γ only for lack of a better nomenclature. The high mobility of the protein fractions during the time of rapid de-

velopment is not confined to calf serum. We have observed the same phenomenon in the serum of young rats and in other cases that will be discussed in a later publication.

Summary. (a) The serum of new-born calves, before the ingestion of colostrum contains no γ globulin and only small amounts of β globulin. (b) During the nursing period the composition of calf serum changes rapidly. The γ globulin appears. Both γ and β increase in concentration and then decrease. The concentration of α and albumin decrease during nursing, with that of the albumin finally increasing. (c) During the period of greatest change in composition of the serum, the protein fractions exhibit an abnormally high mobility.

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Complement-Fixation in Human Pneumonitis with Group-Reactive Virus Antigens.*

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Some time ago we reported the isolation of a virus from 4 cases of severe atypical pneumonia by direct intranasal inoculation of mice.[1] This agent was at first thought to be a variant of psittacosis virus, but subsequent investigations have shown that it is related to a group of viruses which includes not only psittacosis, but also the virus of lymphogranuloma venereum,[2] the virus of meningopneumonitis,[3] and a virus of mouse pneumonia recently isolated by Nigg.[4] The members of this group of agents are characterized by the formation of minute coccoid elementary bodies, by the possession of a common complement-fixing antigen,[5, 6] and by similarities in pathogenicity for experimental animals.

These viruses have been isolated not only from man and birds, but also from ferrets and mice by intranasal passage of lung material. A virus isolated from pigeons by Pinkerton and Swank[7] is apparently very similar to the meningopneumonitis virus.[8] However, this latter agent was shown[1] to differ from the virus isolated by us from cases of atypical pneumonia in its greater virulence for Java rice birds and for mice by the intraperitoneal route, and by its ability to induce a carrier state in mice. Furthermore, antigenic differences between the virus of human pneumonitis and the viruses of meningopneumonitis and psittacosis have been demonstrated by active immunity tests in mice.[9] This, however, does not exclude the possibility that pneumonitis or other respiratory diseases in man may also be caused by psittacotic viruses carried by pigeons[10, 11] or even by similar agents which are carried by animals.[3, 4]

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in coöperation with the California State Department of Public Health.

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4 Nigg, C., *Science*, 1942, **95**, 49.

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8 Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1942, **75**, 575.

9 Beck, M. D., and Eaton, M. D., *J. Inf. Dis.*, 1942, in press.

10 Meyer, K. F., Eddie, B., and Yanamura, H. Y., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 609.

11 Eddie, B., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 291.

TABLE I.
Cross Reactions by Complement Fixation of Human Sera from Cases of Pneumonitis and Lymphogranuloma Venereum.

Serum	Disease	*Complement fixation titers with antigens of		
		Lymphogranuloma venereum	Meningo-pneumonitis	Mouse pneumonia
St.	Pneumonitis†	4/16	8/32	4/8
Li.	”	8/32	16	4/16
Me.	”	4/32	4/16	8/32
Wk.	”	8/8	4/4	4/8
Ca.	”	4/16	4/4	0/16
Go.	”	4/4	2/4	0/4
Lc.	”	4	32	4
Po.	”	16	0	16
Mc.	”	0	0	16
Kl.	”	16	4	16
Pr.	Pneumonitis‡	16	4/16	32
Bc.	”	64	16/64	—
Cr.	”	64	0/32	64
H9	Lymphogranuloma venereum	64	16	16
H10	” ”	32	16	16
H75	” ”	512	—	32
H76	” ”	512	128	16

*In the three columns to the right, numerator is the titer of serum specimens taken early in the disease; denominator the titer of serum specimen taken late in the disease. Where only one titer is given, this is for the late, or convalescent, specimen.

†Unknown etiology.

‡Virus of meningopneumonitis group isolated from sputum.[1]

In view of the apparent ubiquity of this group of agents, it was considered desirable to determine by serological tests how frequently they are etiologically related to respiratory disease. Sera from cases of atypical pneumonia† and from uncomplicated acute upper respiratory diseases were tested by complement fixation with antigens from the viruses of meningopneumonitis, lymphogranuloma venereum and mouse pneumonia grown in chick embryos and using the methods previously described.[2] All tests were controlled with similar preparations from normal chick embryos.

Complement fixation tests also were carried out with sera of immune animals and antigens prepared from the three viruses. Some of the animal sera, especially those from guinea pigs, gave reactions which were relatively specific for one strain. Others gave group reactions, the titers being approximately equal with all three antigens. These sera gave no fixation of complement with antigens prepared from the viruses of influenza or lymphocytic choriomeningitis.

Table I shows some of the significant reactions of human sera with the 3 antigens. Sera from cases of pneumonitis of unknown etiology gave group specific reactions with the viruses of lymphogranuloma venereum, meningopneumonitis, and mouse pneumonitis. Sera from cases definitely diagnosed as virus pneumonia by isolation of a specific virus, and also sera from cases of lymphogranuloma venereum gave similar reactions. Although in some cases there was evidence of specificity for one of the 3 antigens, in general the group specific character of the reaction was quite definite and this has been observed with many other samples of human serum.

Despite the fact that the complement fixation test with this group of viruses was not specific for any one member of the group when applied to human serum, it was considered of some interest to determine what proportion of specimens gave positive reactions. The results with sera from cases of atypical pneumonia and of uncomplicated upper respiratory disease are shown in Table II. In 6 of 70 cases of pneumonitis tested with

† The term atypical pneumonia is used to denote all forms of acute primary bronchopneumonia or pneumonitis without demonstrable bacterial etiology.

TABLE II.
Complement Fixation Tests with Acute and Convalescent Serums.

Disease	Antigen	Number tested	Change in titer associated with illness: No increase, number	Increase 2-fold, number	Increase 4-fold or over, number
Pneumonitis	Meningopn.	70	60	4	6
Upper Resp.	"	80	76	3	1
Pneumonitis	Lymphogran. ven.	61*	43	12	6
Upper Resp.	" "	40	37	2	1
Pneumonitis	Mouse pneumonia	27	19	5	3

* 4 cases showing increase in complement fixation titer with normal yolk sac not included.

TABLE III.
Complement Fixation Tests with Serum Collected During Convalescence Only.

Disease	Antigen	Number tested	Number with titer of: 0	4	8	16 or over
Pneumonitis	Meningopneumonitis	64	30	14	10	10
Upper Resp.	"	76	60	14	1	1
Pneumonitis	Lymphogranuloma venereum	69	30	13	9	17
Upper Resp.	" "	40	29	5	5	1
Pneumonitis	Mouse pneumonia	63	36	10	9	8

the virus of meningopneumonitis the serum collected during convalescence showed a definite increase in titer of 4-fold or greater over the serum taken during the acute phase of illness. Similar definite increases were observed in 6 out of 61 cases tested with the virus of lymphogranuloma venereum and in 3 of 27 cases tested with the mouse pneumonia virus. In other cases increases in titer of lesser degree were observed. One case of upper respiratory disease having the symptoms of influenza showed a definite increase in titer with both antigens. This case gave negative serological tests for influenza A and B. In a few other cases of upper respiratory disease slight increases were observed. Four cases of atypical pneumonia showed an increase in complement fixation titer with normal yolk sac antigen. This emphasizes the importance of normal antigen controls in all tests of this sort.

In Table III are presented the results of tests on cases of pneumonia from which only sera collected during convalescence were available. The titers of sera of patients convalescing from upper respiratory disease are included for comparison. It will be seen that the proportion of serum specimens having titers of 8 or above with the 3 antigens was relatively higher in the cases of pneumonitis than in cases of upper respiratory disease. Titers below 8 are considered normal. In about 15% of the cases of pneumonitis the titers were over 16 or well above normal limits. In many cases the results represent triplicate tests on the same serums with the 3 antigens.

During the past 2 years sputum specimens from 110 cases and lung specimens from 12 cases of atypical pneumonia, and over 100 throat washings from cases of influenza-like illness have been inoculated intranasally into mice with at least 2 subsequent intranasal passages. From only 3 of these specimens‡ (excluding those previously described[1]) was a virus which forms elementary bodies isolated by the method used. The results obtained in these tests, as well as the failure of other investigators to infect mice with sputum from atypical pneumonia, suggest that this pathological syndrome, particularly in its milder form, is rarely caused by a psittacosis-like agent directly transmissible to mice. The possibility that the 15% of cases of pneumonitis

‡ These cases will be described in a later communication.

which gave positive reactions were caused by any one of a group of several related viruses should be considered. Some of these agents may not be directly transmissible to mice.

Summary. Complement fixation tests were done with sera from cases of pneumonitis and acute upper respiratory disease and antigens from the viruses of lymphogranuloma venereum, meningopneumonitis, and the mouse pneumonia virus of Nigg.[4] Positive reactions of a group-specific character were obtained in 10 to 15% of cases of pneumonitis and in about 2% of cases of upper respiratory disease.

Most of the serum specimens tested in this study were obtained through the courtesy of Drs. W. G. Donald, R. T. Sutherland, and Henry Borson, Cowell Memorial Hospital, University of California; Drs. W. J. Kerr and J. W. Brown, University of California Medical School; and Drs. J. Thomas Hardesty, J. E. Walker, and G. B. Hanson, Seaside Memorial Hospital, Long Beach, California.

13878

Detection of Tubercle Bacillus Polysaccharides in Infected Material from Animals and Patients.*

JOHN T. RIORDAN. (Introduced by Francis G. Blake.)

From the Section of Preventive Medicine, Yale University School of Medicine.

In a previous communication[1] a series of precipitin and collodion-particle-agglutination tests has been reported with antigens derived *in vitro* from tubercle bacilli. In view of the extreme sensitiveness of some of these tests, in which positive collodion-particle-agglutination tests were obtained with the tubercle bacillus polysaccharides, in a dilution of one part in ten million, it would seem that, if these polysaccharides exist free within tuberculous lesions, they should be demonstrable there by these methods. It has been our purpose, therefore, to see whether or not this is true; so in the present communication a series of these tests which have been performed on pathological material is recorded.

Materials. A single lot of anti-H37 tubercle-bacillus serum (designated No. 5807A and B) was used as in the former series.[1] † It was prepared by the Mulford Biological Laboratories of Sharp and Dohme in 1925, by the injection of a horse with defatted tubercle bacilli. The immunological properties of this serum have been studied by a number of investigators,[2] including Heidelberger,[3] who found its antibody to be almost entirely of antipolysaccharide specificity. In our own tests,[1] this was confirmed, in so far as positive immunological reactions were obtained with polysaccharide fractions of acid-fast bacilli and with Old Tuberculin, but not with purified protein derivatives of tubercle bacilli.

In the present series of tests, the following material was used as a possible source of antigens which might react with this serum:

I. *Infected embryonic tissue.* Twenty, 9-day chick embryos were implanted with tubercle bacilli (human type), and 9 days later the gross lesions noted on the chorio-allantoic membrane were harvested in 3 eggs.[4] This material was washed in saline, minced,

* Aided by a grant from the John and Mary R. Markle Foundation.

1 Riordan, J. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 622.

† I am indebted in this and in the previous study[1] to Dr. B. Gerstl, of the Department of Pathology, Yale University Medical School, for a supply of this serum.

2 Thomas, R. M., and Duran-Reynals, F., *Yale J. Biol. and Med.*, 1940, **12**, 525.

3 Heidelberger, M., and Mengel, G. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

4 Emmart, E. W., and Smith, M. I., *Public Health Reports*, 1941, **56**, 1277.

TABLE I.
Immunologic Tests for Derivatives of the Tubercle Bacillus in Material from "Infected" Tissue and Patients.

Material	Material treated by: Method A — No. of tests	Method A — Ptn.	Method A — C.P.A.	Special methods B and C — No. of tests	Special methods B and C — Ptn.	Special methods B and C — C.P.A.
Embryonic Tissue	3	—	—	3	+	++
Animal Tissue	10	—	—	4	+	++
Human Pleural Fluid	20	—	—	0	....	....
Human Sputum	8	—	—	10	+	++

— Negative test. + Positive test in all cases tried. Ptn. = precipitin reactions. C.P.A. = Collodion-particle agglutination.

and ground in sand and distilled water for (A) 10 to 20 min, and (B) 60 to 90 min. The ground material was centrifuged at high speed for 30 minutes, the supernatant fluid filtered through a Seitz filter, and in most instances this filtrate was concentrated to approximately 1/10 of original volume *in vacuo* at 50°-55° C.

II. *Tuberculous tissue from monkeys.* This was chosen because of the fulminating type of tuberculous lesions seen in these animals. Tuberculous lung, liver, and spleen were used. Extracts were made by grinding tissue in sand and distilled water (roughly 1 g of tissue to 10 ml of water): (A) 10 to 20 min, and (B) 60 to 90 min. The resulting emulsions were centrifuged, passed through a Seitz filter, and concentrated in much the same manner as the embryonic extracts.

III. *Pleural fluid,* with and without demonstrable tubercle bacilli, from human cases of tuberculosis. This material was centrifuged at high speed, and the supernatant fluid tested.

IV. *Sputum,* freshly collected, and containing 5 to 20 tubercle bacilli per oil immersion field by direct smear, was (A) digested with 3% NaOH at 37° C. for one-half hour. It was then centrifuged at high speed, the supernate neutralized to phenol red with HCl, filtered as above, dialyzed through a cellophane membrane‡ against distilled water at ice-box temperature, and concentrated in the same manner as the embryonic extracts; (B) whole sputum was ground in sand and distilled water for 60 to 90 min, centrifuged, passed through a Seitz filter, dialyzed through cellophane, and concentrated *in vacuo* in the same manner as the tissue extracts; and (C) whole sputum was put in a pyrex tube, stoppered, and immersed in a water bath at 56° C for one week. Smears made of the sputum at this time by the Ziehl-Neelson technique showed some pink-staining rods as well as typical red-staining tubercle bacilli. This material was centrifuged, the supernate saved, and the sediment washed with distilled water 3 times with centrifuging in between, and the supernates pooled. They were then passed through a Seitz filter, dialyzed through cellophane, and finally concentrated by heat *in vacuo* at 50°-55° C.

‡ The cellophane tubing used in these experiments is known as Visking casing, 36/32 inch in diameter.

Results listed in Table I indicate that none of the extracts which were not submitted to prolonged grinding or autolysis yielded serological evidence that tubercle-bacillus polysaccharides existed free in this material (see under columns labelled Method A). Positive results were obtained only with material subjected to Special Methods described above (see under columns labelled Special Methods B and C).

Discussion. In spite of the fact that the polysaccharide fraction of the tubercle bacillus is regarded as the main fraction most soluble in water, it is not easy to demonstrate minute traces of it in tuberculous lesions, or in fluid which may have been in contact with such lesions. Only when the pathological material was subjected to agents that may artificially disintegrate the tubercle bacilli present, did the serological tests yield evidence of the

presence of this antigen.

Conclusion. The polysaccharide fraction of the tubercle bacillus may be liberated in the course of infection produced by this organism, but such fractions are not readily demonstrated by the delicate immunologic methods used in these experiments. The positive results herein recorded have been obtained only in material treated by *artificial* agents which may have disintegrated the tubercle bacilli present.

13879 P

Pituitary and Nervous Control of Chromatic Responses, Especially of Xanthophores, in Killifish (*Fundulus*).

E. F. B. FRIES. (Introduced by A. J. Goldforb.)

From the Department of Biology, the City College, New York, and the New York Aquarium.

Previous work on killifishes has shown the yellow pigmentary effectors (xanthophores) of *Fundulus heteroclitus* and *F. majalis* to be subject to humoral control and to direct, probably double, nervous control permitting independence from melanophore responses.[1] A pituitary hormone dominates the xanthophores of the minnow *Phoxinus*,[2] as it does amphibian melanophores. Pituitary extract causes xanthophore pigment dispersal in this and other teleosts—including *Fundulus*.[3,4] The present paper reports experiments undertaken to verify and evaluate the inferred participation of the hypophysis in pigmentary coördination, with respect chiefly to the xanthophores, in *Fundulus*.

Chromatic responses were determined in 54 *F. majalis* 5 or more days after total hypophysectomy,[5] *i.e.*, when pituitary substances were absent from the circulation. Black-bottomed and white-bottomed vessels evoked darkening and paling, through melanophore change, about as readily as in normal fish (as in *F. heteroclitus*).[6] The hypophysectomized *F. majalis* were clearly incapable of normal adaptive response to yellow, however, irrespective of the duration of their stay over that color (instead of white or pale blue). Yet they became certainly, though only slightly, more yellowish after staying over yellow than otherwise. This fact was verified by reciprocal comparative tests, involving interchange of the yellow and other pale substrates. The xanthophores of the tail fin (as examined microscopically) after hypophysectomy did not effect the usual pigment-dispersing response to yellow, but they did respond to black, thus showing that pituitary intervention is not essential for eliciting their pigment dispersal.

In 20 *F. heteroclitus* the effect of total hypophysectomy on xanthophore response was greater. These fish remained uniformly grayish over a pale ground, whether yellow, white, or blue. Their caudal xanthophores showed none of the normal pigment-dispersing response to yellow (contra Matthews).[3] Nevertheless, these xanthophores could still show the temporary pigment dispersal that may be brought about by handling.

The table summarizes results of keeping normal (N) and pituitaryless (H) fish of both species several hours in bowls of the designated colors.

Pituitary involvement was so manifest in the results of hypophysectomy as to stimulate reconsideration of the question of direct nervous control of the xanthophores. To this end, one or more caudal fin rays were tran-

1 Fries, E. F. B., *J. Exp. Zool.*, 1931, **60**, 389; *Biol. Bul.*, 1942, **82**, 261.

2 Giersberg, H., *Z. vergl. Physiol.*, 1932, **18**, 369.

3 Matthews, S. A., *Biol. Bul.*, 1933, **64**, 315.

4 Abramowitz, A. A., *Am. Nat.*, 1936, **70**, 372.

5 For method, see Abramowitz, A. A., *Science*, 1937, **85**, 609.

6 Reviewed by Parker, G. H., *Proc. Am. Acad. Arts Sci.*, 1940, **73**, 165.

		White	Pale blue	Yellow	Black
Dorsal aspect of fish	N (both species)	Pale; not yellowish	Pale; not yellowish	Pale; markedly yellowish	Dark
	H (*majalis*)	Pale; not yellowish	Pale; not yellowish	Pale; slightly yellowish	Dark
	H (*heteroclitus*)	Pale; not yellowish	Pale; not yellowish	Pale; not yellowish	Dark
Caudal xanthophore pigment	N (both species)	Concentrated	Concentrated	Moderately dispersed	(Often much) dispersed
	H (*majalis*)	Concentrated	Concentrated	Concentrated (?)	Dispersed
	H (*heteroclitus*)	Concentrated	Concentrated	Concentrated	

sected in 45 of the *F. majalis* and 19 of the *F. heteroclitus*, mostly 11 or more days after hypophysectomy. Without exception in *F. majalis* (kept over pale blue or white) the caudal bands thus denervated acted as in normal fish; *viz.*, they turned both darker and yellower through pigment dispersal in their chromatophores. These bands resembled those of unhypophysectomized fish also in subsequent fading and other changes by which they differed characteristically from innervated sectors of the tail. In *F. heteroclitus*, too, the pituitary was found dispensable for xanthophore response characteristic of denervated caudal bands.

Intraperitoneal implantation of fresh *Fundulus* pituitaries (3-6 per recipient) into 7 individuals of each lot, 5-60 days after hypophysectomy, induced pigment dispersal in the xanthophores and melanophores of the faded denervated bands. It did so regardless of the substrate color. In the xanthophores of innervated parts of the fin, the implantation made possible normal dispersal in response to yellow and it opposed, without abolishing, their pigment-concentrating response to white or blue.

Evidently, a pigment-dispersing pituitary secretion, presumably intermedin, is one of several factors controlling xanthophore changes in *Fundulus*. It must be the predominant factor in *F. heteroclitus* while not preponderant in *F. majalis*. Moreover, the results (including further details) confirm the conclusion (1942)[1] that the xanthophores are directly innervated, probably by pigment-dispersing as well as -concentrating fibers. The system of mechanisms regulating the xanthophores resembles that governing the melanophores; in both species, however, the melanophores are more completely under the sway of their direct innervation.

13880

The Quantity of Thrombin Required to Clot Heparin-Plasma Mixtures.*

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It has been shown[1] that approximately 2.5 units of thrombin are required to cause clotting of 1.0 cc of oxalated bovine plasma in 15 sec. If heparin be added, larger amounts of thrombin are needed to do this, and we shall show that, within certain limits, the amounts needed vary in direct proportion to the amounts of heparin added. This linear relationship between heparin and thrombin provides a simple and reliable basis for the assay of heparin.

Materials and Methods. Crystalline sodium salt of sheep heparin was obtained through the courtesy of Dr. L. B. Jaques of Toronto. On the basis of his analysis it contained 7% water of crystallization, and possessed 26 anticoagulant units[2] per mg (cat assay). It was dissolved in saline (0.9% NaCl).

Fresh beef blood was collected at the slaughter house. Seven parts of blood were mixed with 1 part of 1.85% solution of potassium oxalate, and then centrifuged to obtain the plasma. Such plasma can be stored at —40° for more than a week, without alteration in the properties for which it is used.

Thrombin, free of antithrombin, was prepared by methods previously described.[3] It was dissolved in 0.9% salt solution. Assay was carried out by a simplified technic which involves treating 0.9 cc quantities of oxalated plasma with 0.1 cc quantities of thrombin solution of varying dilution. The tube which clots in exactly 15 sec contains approximately 2.5 units of thrombin.[1] From this and from the known dilution, the unit strength of the original stock solution is readily derived.

Experiments. A series of clotting mixtures was prepared, containing oxalated plasma, together with variable amounts of heparin. These mixtures were then clotted with thrombin. When heparin was omitted altogether, 2.9 units of thrombin were required to cause clotting of 1 cc of the mixture in 15 sec (Fig. 1). On addition of progressively stronger solutions of heparin, it was necessary to add stronger thrombin solutions in order to maintain the standard 15-sec clotting interval. As shown in the chart, the balanced relationship between thrombin and heparin concentration is a straight line function, and can be expressed by the equation

$$Y = 2.89 + 280X$$

This experiment has also been carried out with oxalated plasma of man, dog and pig. In all cases, a linear relationship prevailed. The slope and the intercept on the Y axis were found to vary, however.

Discussion. It must be assumed that the addition of heparin to clotting mixtures brings about destruction of considerable amounts of thrombin long before clotting is complete. In several experiments we have added thrombin in smaller amounts. In one case a clotting interval of 20 sec was selected. The balanced relationship between heparin and thrombin concentration was then not a straight line. In such an experiment heparin and its co-factor have more time to destroy thrombin. It thus appears that the linear relationship is not valid throughout the entire range of experimentation. However, under restricted conditions, as shown in Fig. 1, the linear relationship does hold, and for many purposes it is of great value in the assay of heparin. In conducting such an assay, one prepares a stock thrombin solution

* Aided by a grant from the John and Mary R. Markle Foundation. Funds were also supplied by the Graduate College, State University of Iowa.

1 Seegers, W. H., and Smith, H. P., *Am. J. Physiol.*, 1942, **137**, 348.

2 Jaques, L. B., and Charles, A. F., *Quart. J. Pharm.*, 1941, **14**, 1.

3 Seegers, W. H., *J. Biol. Chem.*, 1940, **136**, 103.

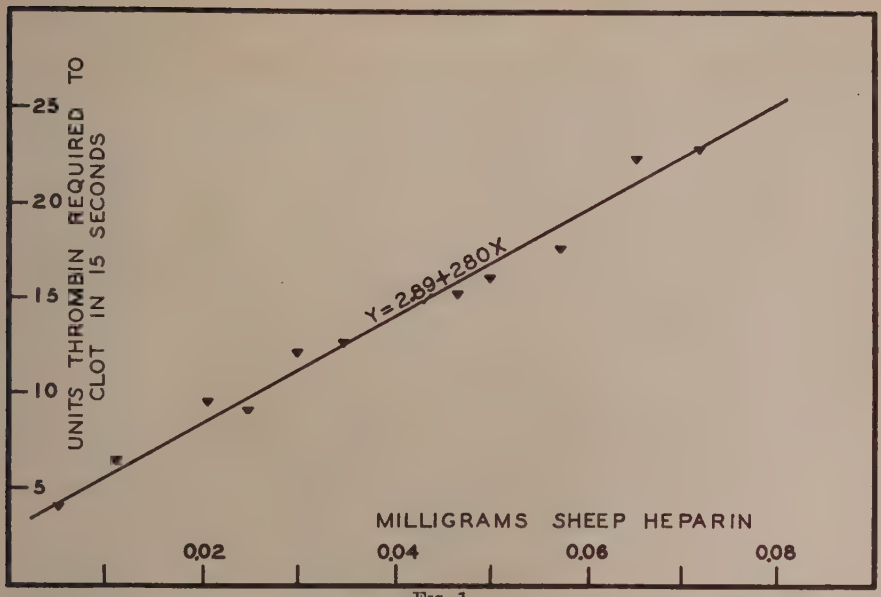

FIG. 1.

To a tube containing 0.8 cc of oxalated plasma were added 0.1 cc of saline (0.9% salt solution) and 0.1 cc of thrombin solution, dissolved in saline, and containing a total of 2.9 units of thrombin. Clotting occurred in exactly 15 seconds. To other tubes 0.8 cc of oxalated plasma was added, together with 0.1 cc of heparin solution of progressively increasing concentration. At each heparin level 0.1 cc of thrombin solution of variable concentration was added. By repeated trial, it was possible to determine the number of units of thrombin needed to cause clotting in exactly 15 seconds. This was done at a room temperature of 26-28°C. The amounts of thrombin in the final clotting mixture are plotted against the corresponding amounts of heparin.

of known titer, somewhat in excess of 200 units per cc. To 0.8 cc oxalated plasma 0.1 cc of the heparin in question is added. The mixture is then clotted with 0.1 cc of the thrombin solution at a room temperature of 26-28°C. By successive trial, and by interpolation, one then adjusts the dilution of the heparin or of the thrombin in such a way that a clotting time of exactly 15 sec is obtained. Knowing the number of thrombin units added, one can readily calculate the heparin concentration from a chart of the type shown in Fig. 1. As a control, a standard solution of heparin should also be analyzed. If necessary, slight corrections can then be made in the value obtained.

Summary. The amount of antithrombin-free purified thrombin required to clot plasma in 15 sec is, within limits, directly proportional to the quantity of heparin added to the plasma. This relationship can be used for the assay of heparin.

13881 P

A New Agent for Objective Measurement of Circulation Time.

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The arm-to-head circulation time may be objectively measured by the injection of 1 cc of aminophylline (0.24 mg) into the antecubital veins. The end-point of the reaction is signalled most frequently by a marked increase in the depth of inspiration, which in many cases amounts to a gasp. While this occurs in every case, in some instances it may be preceded by swallowing movements, a change in facial expression resulting from subjective sensations, or a sharp catch during expiration. The first observable change is taken as the end-point.

The test was tried in 92 patients at various stages in the postoperative state whose ages ranged from 12 to 82 years. Only once in 92 trials was any difficulty experienced in recognizing the end-point. The circulation times ranged from 7.1 to 20.4 seconds averaging 12.4 seconds.

The test is safe, the end-point is sharp, the method is objective, the amount injected is small, the agent is readily accessible. All of these factors combine to commend it.

13882

Studies on the Microcataphoresis of Animal Parasites.

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Extensive studies have been made on the electric charge of bacteria and suspensions of most of viruses which have been found to carry a negative charge. The amount of the current as measured in milliamperes is primarily responsible for the accumulation and destruction of these microorganisms at the positive pole. With viruses it has been found that the nature of the charge influences passage through a filter, so that this characteristic has been utilized to free these filter passers from contaminating material. Moreover, the knowledge of the charge of the organism is important in devising methods for its destruction *in vivo* and *in vitro*.

Description of Apparatus. In principle and in many structural respects the cataphoresis cell constructed was essentially like the original one devised by Brown and Broom.[1] We merely superimposed a bored-glass section communicating with the channel and a brass plate with appropriate apertures into which the chamber holding the zinc sulphate solution and microorganism suspension could be cemented.

Materials employed. Trypanosoma cruzi, Leishmania brasiliensis and *Leishmania tropica* were grown on Senekjie's medium.[2] The cysts and eggs of intestinal parasites were concentrated by the zinc sulphate technic of Faust *et al.*[3] The filariform larvae of

[1] Brown, H. C., and Broom, J. C., *Brit. J. Exp. Path.*, 1929, **10**, 61.

[2] Senekjie, H. A., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1939, **33**, 267.

[3] Faust, E. C., Sawitz, W., Tobie, J., Odom, V., Peres, C., and Lincicome, D. R., *Parasitol.*, 1939, **25**, 241.

Strongyloides were cultured from the feces of a chimpanzee, the larvae of *Trichinella spiralis* were hatched in artificial gastric juice, and *E. histolytica trophozoites* were grown on egg-liver extract medium.

Five *T. cruzi* strains were tested. When current A (*i.e.* 90 volts and 7 milliamperes) was applied to culture trypanosomes in the cell, there was immediate increased motility with a drift towards the positive pole, a tendency to clumping which settled to the bottom of the chamber. Motility steadily diminished, ceased in 8 minutes and the organisms disintegrated in 10 minutes. The armadillo strain was more resistant. When an arthropod strain was treated with 0.3% formalin for 24 hours distintegration was incomplete in one hour. When grown on leishmania medium containing 2% lithium chloride to produce rough forms, complete disintegration took place in 17 minutes. Trypanosomes from the rectum of *Triatoma infestans* survived about 30 minutes and disintegrated very slowly. *L. brasiliensis* and *L. tropica* differed very little from *T. curzi* except that they were more sensitive and death and disintegration took place in 8 minutes.

With current A, *E. histolytica* trophozoites migrated to the positive pole. In 15 seconds they became actively motile, ceased moving in 45 seconds and exploded in 3 minutes, leaving no trace. The rounded precystic forms from cultures were similarly charged, but remained nonmotile, showed hyalinization and disintegrated in 2 hours. Concentrates of the cysts of *Giardia lamblia, Endolimax nana, Chilomastix mesnili, E. coli* and *E. histolytica* were resuspended in normal saline and zinc sulphate solution of sp gr 1.18, and were tested with current A. All were negatively charged, showed slow hyalinization, and even after 3 hours a considerable number remained intact and apparently viable. There was no excystation. With current B (*i.e.* 350 volts and a constant but unknown milliamperage) hyalinization took place sooner.

The eggs of *Ascaris lumbricoides, Trichocephalus trichiurus* and *Ancylostoma caninum* were concentrated and floated in zinc sulphate solution. With current A no changes were observed, but with current B they drifted to the positive pole. *T. trichiurus* eggs showed no changes but the other two showed hyalinization and shrinkage of the embryos. Oocysts of *Isospora canis* remained unchanged with current A, but with current B they drifted to the positive pole, and the enclosed spores showed shrinkage in 2 hours.

Filariform larvae of *Strongyloides* suspended in normal saline became actively motile and migrated to the positive pole. Motility progressively diminished and eventually all motion ceased, hyaline changes took place in 45 minutes, the worms clumped in masses and death took place in 2 hours. The free-living adult females showed the same active motility, but hyaline changes took place in 10 minutes. The uterus was contracted and the eggs were seen to be extruded from the uterine pore. All the eggs showed hyaline changes. All motility ceased in 45 minutes. With current B the larvae manifested marked contractions and were killed in a few minutes, but they drifted to the positive pole.

Larvae of *T. spiralis* suspended in normal saline coiled and uncoiled, manifested active movements and drifted to the positive pole. Motility progressively diminished, all movements ceased in 20 minutes and finally they coiled up. When the current was broken, they slowly uncoiled and again developed motility. All motility ceased in 2½ hours and the worms relaxed. With current B there was active motility, but the worms died in 15 minutes in a relaxed position.

Conclusion. T. cruzi, L. brasiliensis, L. tropica, E. histolytica, E. coli, G. lamblia, E. nana, C. mesnili, I canis, A. lumbricoides, T. trichiurus, A. caninum, T. spiralis and *Strongyloides* are all negatively charged. Since bacteria and *E. histolytica* are both negatively charged, it is not possible to purify cultures of the latter by electrolytic methods.

13883

Thirteen Years of Homologous Function in Normal and Supernumerary Grafted Limbs.

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For nearly twenty years it has been known that when a supernumerary limb in urodele amphibians is grafted adjacent to a normal limb, so that it acquires innervation from a portion of the normal limb plexus, the corresponding muscles of the two limbs always contract synchronously and with the same degree of intensity. This phenomenon has been called "homologous function" by Weiss who, in order to explain the mechanism, proposed the so-called "Resonance Theory" of reflex activity.[1]

In his earlier experiments, Weiss grafted fully differentiated limbs (fore and hind) of *Salamandra maculosa* larvæ close to the normal. In making the wound for the insertion of the limb, one or more of the normal limb nerves were cut. These were found to regenerate into those muscles of the orthotopic limb which were rendered nerveless by the nerve section, as well as into the denervated muscles of the supernumerary limb. Weiss pointed out that there occurred an "at random" regeneration of the nerves, consequently homologous function could not be explained upon any structural specificity in regenerating nerve pathways.

Homologous function has been described also by the author[2] in limbs that have been grafted as embryonic rudiments. In these cases, the normal forelimb rudiment of Amblystoma embryos was excised and grafted 3 or 4 segments caudal to the normal position. The wound was left uncovered—thus providing for regeneration of a limb in the original site (Fig. 1). Under such conditions one might obtain 3 limbs, *viz.*, a regenerant and a double limb arising from the graft. When this occurred, synchronized movements of the homologous muscles ensued; regardless

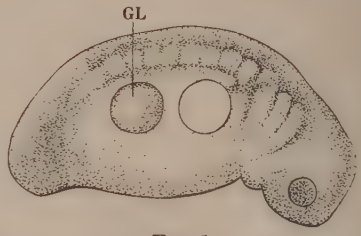

Fig. 1.
Drawing of Amblystoma embryo (stage 28) showing anterior limb rudiment (GL) grafted 4 segments caudal to the normal position. The clear disc represents the uncovered wound, from the margin of which a limb regenerated. × 10.

of the peripheral nerve pattern, provided all 3 limbs received some innervation from the brachial region of the cord (third, fourth, and fifth spinal segments). Homologous function was found not to depend upon the distribution of any single brachial nerve to 2 or more limbs. In some cases the limbs were supplied entirely by separate brachial nerves.

The case reported in this communication is of interest mainly because it was kept under observation for 13 years. In the fall of 1929, I obtained several batches of fertilized eggs from adult axolotls in the laboratory. In Harrison's stage 29 (tail bud stage), numerous limb grafts were made in the manner described above.

In many cases the grafted limb reduplicated and a limb regenerated in the normal site. The case shown in Fig. 2 illustrates the condition. This animal exhibited synchronized movements in the homologous muscles after the limbs became functional, and the muscles continued to function in

[1] Although Weiss has published many papers in various journals on the phenomenon of homologous function and the "Resonance Theory," the reader, if unfamiliar, is referred to his review paper in *Biological Reviews*, 1936, **2**, 494-531, for a general account of the facts, a discussion of the theory, and bibliographic references to his earlier work.

[2] Detwiler, S. R., *J. Exp. Zool.*, 1925, **38**, 461.

this manner without any change during the thirteen years under observation. A gross dissection of the brachial nerves showed that the third and fourth spinal nerves supplied

FIG. 2.

Ventral view of preserved axolotl (*A. mexicanum*) showing three limbs on the right side. Limbs 2 and 3 are mirror image reduplicants developed from a graft as shown in Fig. 1. Limb 1 regenerated from the wound as indicated in Fig. 1. The animal when fixed was 13 years old and measured approximately 9 inches in length.

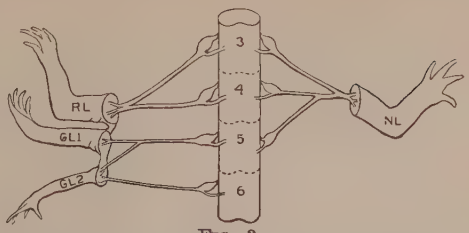

FIG. 3.

Diagrammatic representation of the nerve supply to the limbs shown in Fig. 2. The regenerated limb (RL) is supplied by the third and fourth (brachial) nerves. The two members of the grafted reduplicant (GL1 and GL2) are supplied by the fifth (brachial) nerve. The sixth (post brachial) nerve also enters the posterior member of the reduplicant. The normal (left) limb (NL) is supplied by the third, fourth and fifth (brachial) nerves.

the regenerated limb (RL). The two members of the reduplicated grafted limb (GL1 and GL2) were supplied by the fifth nerve (Fig. 3). The sixth nerve also supplied the posterior member of the reduplicant, but it is known from previous experiments[3,4] that supernumerary grafted limb muscles when supplied by spinal nerves below the brachial region fail to exhibit coördinated or homologous movements.

The case herewith described offers proof that when the nervous pattern for homologous movement is once established, it continues to function without change—a condition which would be expected. It is of interest to point out also, that homologous function in the muscles of a normal and an adjacent supernumerary limb does not depend upon the distribution of the same nerves to the two limbs, nor does it depend upon any peripheral nerve communications between the brachial nerves (Fig. 3). The origin of the nerves from the original brachial region of the cord is the essential condition regardless of their peripheral distribution to 2 or even 3 limbs.

[3] Detwiler, S. R., *J. Exp. Zool.*, 1920, **31**, 117.

[4] Detwiler, S. R., and Carpenter, R. L.; *J. Comp. Neur.*, 1929, **47**, 427.

13884

Observations on Occurrence of Lipemia in Rats with Nephrotoxic Nephritis.

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In previous studies[1,2] on the chemical disturbances occurring in rats with nephritis induced by the injection of nephrotoxic sera, we noted that a gross lipemia was frequent during the first 2 weeks of the disease while the animal exhibited edema, hypoproteinemia, and albuminuria. In the course of other studies some additional observations were made on nephrotoxic rats to determine the uniformity and severity of lipemia in this experimental disease. These are reported here.

Experimental Procedure. Rats with severe acute nephrotoxic nephritis which had been employed as controls in immunological studies, or for titration of nephrotoxic sera,[3] were sacrificed by cardiac puncture 4 to 18 days after receiving the last of a series of several daily injections of anti-rat-kidney serum. The oxalated blood from these animals was centrifuged and the plasma analyzed for lipids by the method of Kirk, Page, and Van Slyke.[4] Whole blood urea nitrogen was determined by a modification of the gasometric hypobromite method of Van Slyke and Kugel.[5]

Results. Blood from each of the rats in the edematous phase of acute nephritis appeared lipemic on inspection and on chemical analysis, the total lipid carbon concentration ranged from 916 mg % to 8660 mg % (see Table 1). Total cholesterol, ranging from 208 mg % to 871 mg %, was regularly elevated in roughly the same proportion as the total lipid carbon. The total lipid phosphorus determined on some of the bloods exhibited a wide range of values. There was no apparent correlation between the total lipid carbon and the degree of renal failure as measured by increase of blood urea nitrogen. Gross lipemia bore some relationship to the intensity of the acute nephritis, however, for it was not observed in non-edematous rats with presumably less severe renal involvement. Total plasma lipid carbon in two normal rats of the same strain averaged 559 mg %. Hansen and Brown[6] using a similar extraction procedure but determining total fatty acids and cholesterol by Bloor's methods found that in their normal rats the total lipid carbon averaged about 300 mg % and the cholesterol about 90 mg %. These values are somewhat below the 2 normal rats we observed and serve to emphasize further the magnitude of the lipemia observed in nephrotoxic nephritis.

Comment. The elevation of plasma lipids in all edematous rats with severe acute nephritis induced by anti-kidney serum indicated that hyperlipemia is a regular feature of an early stage of this experimental disease. Gross lipemia disappeared in the second or third week along with anasarca when the rat survived, but plasma protein values did not rise to a normal range until the fourth or fifth week;[2] furthermore, in our experience terminal chronic nephritis, often characterized by plasma protein levels as low as those encountered in the acute phase, has not been associated with gross lipemia. These findings suggest that the lipemia in nephrotoxic nephritis may be related to some acute tissue injury, either renal

1 Smadel, J. E., and Farr, L. E., *J. Exp. Med.*, 1937, **65**, 507.

2 Farr, L. E., and Smadel, J. E., *J. Exp. Med.*, 1939, **70**, 615.

3 Swift, H. F., and Smadel, J. E., *J. Exp. Med.*, 1937, **65**, 557.

4 Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1934, **106**, 203.

5 Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, 1933, **102**, 489.

6 Hansen, A. E., and Brown, W. R., *J. Nutrition*, 1937, **13**, 351.

TABLE I.
Plasma Lipid Concentration in Nephrotoxic Rats.

Rat No.	Day of disease	Total lipid carbon, mg per 100 cc	Total cholesterol, mg per 100 cc	Total lipid phosphorous, mg per 100 cc	Blood urea nitrogen, mg per 100 cc
1	18	2410	253	23.0	13
2	6	1988	387	5.3	11
3	9	2768	473	29.8	34
4	7	1118	208	5.0	34
5	6	1552	218	—	73
6	7	2385	248	—	25
7	12	6413	856	—	40
8	11	8660	713	—	41
9	7	2384	871	—	64
10	5	4325	779	—	46
11	4	916	223	—	—
12	4	1155	378	—	—

or otherwise, and that it is unlikely to be a compensatory osmotic mechanism for the lowered oncotic pressure due to hypoproteinemia.

Summary. Significant hyperlipemia was regularly encountered in rats with severe acute nephritis induced by anti-kidney serum.

13885

Significance of Salmonella in Ulcerative Cecitis of Rats.*

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Chronic ulcerative cecitis of rats is an ideal disease for chemotherapeutic and prophylactic tests. Unlike most acute experimental infections of small laboratory animals cecitis progresses gradually over a period of months and affords an opportunity to study the effect of drugs on a chronic low grade infection. It seems important therefore to determine definitely the nature of the agent which causes this disorder. Buchbinder and his associates[1] concluded that cecitis of rats was an enzoötic form of paratyphoid infection. Their view was based on the isolation of *S. enteritidis* from the stools and from the spleen as well as on the presence of agglutinins. They give no details of the cultural reactions of their strains of salmonella, of the agglutinin titers, or of the exact relation of salmonella to the lesions. Nor is it clear whether they dealt with a single specific strain.

While these observations are striking they do not actually prove that salmonella is the primary etiological agent of rat cecitis. Stewart and Jones[2] pointed out, for example, that paratyphoid infection or infestation of both laboratory and wild rats is common and that various strains are encountered. Furthermore, Buchbinder and his group report no observations on the experimental production of cecitis with strains of salmonella, and finally the exquisitely chronic and localized lesions so well described and pictured by Stewart and Jones bear no clinical resemblance to any known paratyphoid infection.

* Supported by a grant from the Rockefeller Fluid Research Fund.

1 Buchbinder, L., Hall, L., Wilens, S. L., and Slanetz, C. A., *Am. J. Hyg.*, 1935, **22**, 199.

2 Stewart, H. L., and Jones, B. F., *Arch. Path.*, 1941, **31**, 37.

Our experiments were done in the attempt to find out more about the relation of salmonella to rat cecitis.

1. *Isolation of Salmonella from Rat Cecitis.* Cultures were made of cecal contents obtained at autopsy at various times from 12 rats with advanced cecal disease. The material was plated on McConkey agar plates (Difco). Suspicious colonies were fished, subcultured in various carbohydrates and tested for agglutination by stock sera. An attempt was made to estimate the proportion of salmonella in relation to "colon" bacilli. Salmonella were isolated in every case, usually as the predominating organism. Furthermore, the carbohydrate reactions were always the same and every strain was agglutinated in high titer (1:300 up to 1:1280) by a single specific anti-serum. It seems established then that in this epizoötic at least a specific strain of salmonella is intimately associated with the lesions.

In 4 cases of advanced "cecitis" cultures were also made of fluids aspirated from the cyst-like collections which are found between the coils of intestine adherent to the diseased cecum. No salmonella or other organisms were obtained from these fluids. In three rats enlarged glands lying adjacent to the cecum were carefully dissected away with sterile precautions under ether anesthesia. Cultures were made by grinding the glands with sand and broth and planting on a variety of liquid and solid media. In contrast to the cyst fluid all yielded the same type of salmonella as that obtained from the stool cultures, and the organisms were agglutinated by the same specific serum.

Having established, then, the invariable association of a specific organism with the

FIG. 1.
Sections of intestine from Rat No. 32 with fulminating enteritis.

TABLE I.
Results of Feeding Salmonella.

Rat No.	Wt at start of exper., g	Wt at end of exper., g	Gain, g	Cecitis
		Experimental Animals.		
43	66	166	100	0
45	66	206	140	0
97	52	192	140	0
55	66	174	108	+
57	70	204	134	+
01	52	178	126	+
59	70	204	134	++
87	72	218	140	++
07	52	218	166	++
61	64	190	126	+++
		Controls.		
44	56	144	88	0
46	62	154	92	0
56	62	166	104	0
72	56	174	118	0
96	52	206	154	0
88	60	200	140	0
70	62	194	132	+
86	52	150	98	+
02	62	168	106	++
04	58	170	112	++
08	52	148	96	++
98	50	190	140	+++

0 = No cecitis.
+ = Superficial erosion not over 0.5 cm in diameter.
++ = Shallow ulcer 0.5 to 1.0 cm with pericecal adenitis.
+++ = Ulcer over 1 cm in diameter with marked pericecitis.

lesion of "cecal disease," it became of importance to know whether this organism was also present in contacts without clinical lesions. At odd times cultures had been made from cecal contents of rats showing no cecitis; in no case was salmonella isolated, and for the most part there appeared a normal growth of *B. coli*. However, a more conclusive experiment was done. A group of normal rats, 5 to 6 months old, which had been housed in the same cages since birth were sacrificed under ether anesthesia. In this group advanced cecitis was found in 5 rats; hence the other 8 in whom no lesions could be discovered had been in intimate contact with the infected animals. Cultures from the cecal contents of these normal rats yielded no salmonella at all in 6 instances. In a seventh normal rat one colony of *S. enteritidis*, agglutinated 1:320, was isolated, and in the eighth animal 2 colonies were recovered which were agglutinated up to 1:640. However, inasmuch as the entire environment was contaminated these occasional colonies can hardly be considered very significant, and it may be concluded for all practical purposes that animals without cecitis even when in contact with others with advanced disease were not infected with the specific organism.

Intimacy of the association of the specific salmonella with the *lesions* of cecitis is further shown by a comparison of cultures of stool and cecal material. A whiff of ether given to a diseased rat led to the expulsion of a pellet of stool which was caught in a test tube for culture. The animal was immediately anesthetized and cultures were also made from the cecal contents. In the case of four animals which had cecitis salmonella was isolated in each instance from the cecal contents; in three of these no salmonella could be recovered from the stool, and in the fourth only a very few colonies, although the cecal contents yielded nearly a pure growth.

The above experiments suggest strongly that a specific strain of salmonella was the cause of cecitis in this epizoötic. It seemed important, however, to study the effects of

introduction of the organism into healthy rats. The obvious difficulty here was the knowledge that the "spontaneous" incidence of cecitis in the colony was nearly 100%. The problem of control was therefore paramount.

2. *Attempts to Produce Cecitis by Ingestion of Strains of Salmonella Isolated from Cecal Contents.* In a first group of experiments huge amounts of culture were placed in the water bottles of healthy rats. Within 48 hr all come down with a violent diarrhœa and approximately half the animals died. Autopsy showed an extreme diffuse acute enteritis with watery bowel contents from which the specific salmonella was recoyered in pure culture. An example of the lesion is shown in Fig. 1. Five out of a group of 12 rats recovered from the acute infection and remained apparently well. However, when they were sacrificed 10 weeks later 3 of the 5 showed typical advanced ulcerative cecitis.

In a second group of experiments small amounts of 24-hr agar culture of the salmonella (one or two loops) were placed in the water bottles nearly every day over a period of approximately 2 months. The animals remained well with the exception of 2 who had transient diarrhœa. There were 12 animals in the experimental group and 12 litter mate controls who received no culture. When the animals were sacrificed (Table I) the incidence and severity of cecitis was essentially the same in the two groups. The slight differences as shown by many observations in this laboratory are not significant.

Hence it can be concluded that the continuous ingestion of huge doses of salmonella over and above the minimal numbers which were prevalent in the laboratory did not serve to increase significantly the incidence of cecitis or to make it appear earlier in the experimental group. If the dose was pushed beyond a certain point there developed not chronic ulcerative cecitis but acute enteritis—an entirely different lesion. Furthermore even in rats with the most advanced cecitis associated with heavy infestation of salmonella severe acute diffuse enteritis never developed spontaneously.

Summary. It appears, then, that no proof has been brought that salmonella is the cause of ulcerative cecitis of rats. In our laboratory a specific strain of salmonella was, to be sure, intimately associated with the lesions and this organism further was shown to be pathogenic for rats, since an acute diffuse enteritis was readily produced and the organism was again recovered from the lesions. Chronic cecitis, however, failed to develop to a significantly greater extent than in untreated controls. It is possible that some other agent is the primary cause of cecitis and that salmonella is merely an associated organism or one which tends to act as a secondary invader. Further search for such a primary agent is in progress.

We are indebted to Miss Gail Reeves for assistance with the cultures.

13886

Attempts to Transmit Human Infectious ("Benign") Epitheliomatoses to Rabbits.

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The transmission of human infectious ("benign") epitheliomatoses to animals is a problem as yet unsolved. Judging from the literature, there have been no positive results since the single experiment of Ullmann,[1,2] who succeeded in transmitting the virus of highly infectious papilloma laryngis to the mucous membrane of the genitals of a female dog and, at the same time, to the skin of his own arm.

Ullmann concluded from his experience that rodentia are not susceptible for the human virus of epitheliomatoses. Shope[3] described the infectious papillomatosis observed in wild cotton rabbits and was successful in transmitting this virus-induced neoplasm not only to cotton tails but also to domestic rabbits.

Therefore, the possible susceptibility of rodentia for human infectious epitheliomatoses again became of interest. The viruses of both animal and human papillomas and warts are epidermotropic; both neoplasms consist of epidermal cells infected with the virus.

In our experiment we tried to determine the susceptibility of rabbits for the human virus by using varying methods as well as varying sites of inoculation. The technic of Shope was included. Fresh tissue emulsions were inoculated in female rabbits of about 2 months of age, as follows:

(1) Excised finger warts of 2 juvenile female individuals, obtained through the kindness of Doctor Caroll S. Wright in Philadelphia, were ground in a small amount of saline, and 0.1 to 0.2 cc of this suspension was inoculated in 4 rabbits as follows: (a) intracutaneous injection in the ears and the shaved abdominal skin; (b) intramucous injection and (c) scarification followed by rubbing in the suspension in the mucous membrane of the genitals; (d) implantation in a superficial pocket of the genital mucous membrane of one small tissue particle.

(2) Particles of papilloma laryngis of 4 children, obtained through the kindness of Doctor Chevalier L. Jackson of the Bronchoscopic Clinic of Temple University, Philadelphia, were inoculated as a thick emulsion in 0.1 to 0.2 cc of saline in 5 rabbits; (a) by intracutaneous injection in the ears and the abdominal skin; (b) by scarification of the shaved abdomen followed by dropping on the emulsion and rubbing in with a metal spoon (Shope); (c) by intramucous injections, part in the lips and gums, part in the genitals.

The animals were examined every 7 days over an observation period of 4 to 6½ months (October 4, 1941 to April 21, 1942). Pieces of the skin and the mucous membranes, taken from the sites of inoculation, were excised for histological examination. Although all results were negative, we consider a brief report worth while in the interest of further investigation.

1 Ullmann, E. V., *Acta oto-laryngologica*, 1923, **5**, 316.

2 Ullmann, E. V., and Cornwallis, Or., *Dermatol. Wachenschrift*, 1930, **91**, 1864.

3 Shope, Richard E., *J. Exp. Med.*, 1933, **58**, 607.

13887

A Technic for Appraising Ability to Resist Gravitational Shock in the Rabbit.

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The day to day and hour to hour changes in ability to resist the pull of gravity against the venous return to the heart are of importance not only to the clinical problem of orthostatic hypotension and to the tactical problem of pilot failure under combat conditions but also to the more general problem of maintaining health and efficiency at occupations requiring prolonged, unrelieved sitting or standing. Tests have been proposed for the recognition of impairment and exploratory information gathered as to the extent of effect contributed by infection, lack of exercise and fatigue.[1-6] Little has been done in the way of quantitative appraisal of the possibilities for correction, stabilization and control. Mechanical aids have been suggested,[1,7,8] and vasoconstrictive drugs.[7-9]

The upright position imposes gravitational restrictions on circulation regardless of species.[10] Rabbits accustomed to small cages become markedly affected by enforced standing.[11] Circulatory interferences are established sufficient to cause a 30 to 100% decrease in ability to warm up after chilling. The effect is adapted to quantitative measure[12] and is shown below to be modified by infection and by lack of exercise in the same way that has been observed for man. The converse effects of ascorbic acid and desiccated thyroid also are described as instances of a use of the method for further exploration.

Method. The rabbits were dropped into water held at 66°F and allowed to swim freely until the rectal temperature had fallen to a point near 95°F. They were then removed from the water and dried. The course of the subsequent temperature rise was determined and recorded as indicated in Fig. 1, using identical clinical thermometers inserted to a depth of 2½ inches for 1½ min. When the temperature had risen to a point near 96.5°, the rabbits were placed in boxes enforcing an erect, vertical position for one-half hr following which serial temperature measurement was resumed until 100° had been passed. The completed temperature records were examined for the number of degrees rise accomplished during the half hour of enforced standing. A rise of 0.2° was considered to be indicative of a greater degree of interference with circulation, during the half hour of enforced standing, than a rise of 2.0°, etc.

The highest rise observed was 4.0°, in an exceptional rabbit, following preliminary hardening. The lowest was —0.9°, in a rabbit 4 years old. All of the rabbits were in sufficiently good condition[12] to make a rise of 2 to 4°, in one-half hour, when free of the gravitational stress of the upright position.

Reproducibility. Spontaneous changes, possibly associated with week to week changes in health, occurred to an extent re-

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8 MacLean, A. R., and Allen, E. V., *J. Am. Med. Assn.*, 1940, **115**, 2162.
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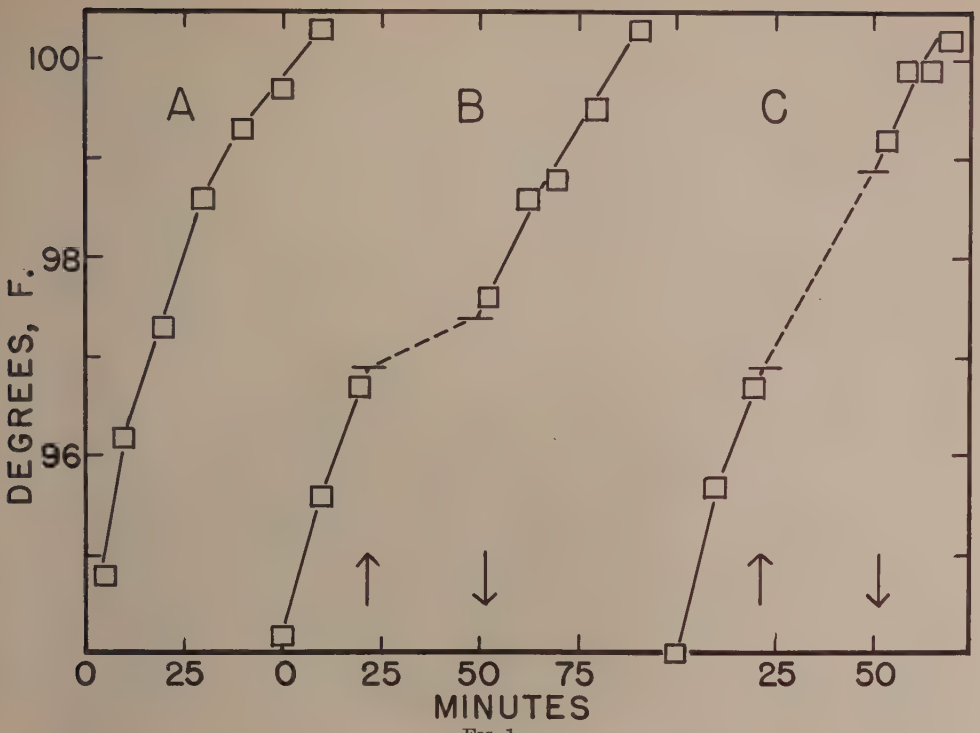

FIG. 1.

A typical record of the rate of temperature recovery after chilling in a rabbit (A) kept in the normal horizontal position throughout the test and (B and C) forced to stand upright for the half hour indicated by the arrows. The rabbit had not been chilled during the week prior to test B and had been chilled on each of the two days preceding test C. The chillings were given as an incentive to exercise (shivering, etc.).

quiring the use of groups large enough to give a stable average (Table I).

Infection. Rabbits so commonly harbor low-grade infections and infestations that freedom from such handicap cannot be assumed even when no evidence of snuffles, wet adherent feces, ear or skin conditions, abscesses, or periods of elevated temperature or weight loss is apparent. Those of the rabbits in Table I with manifest evidence of infection were, on the whole, the least able to endure enforced standing.

Exercise. The most effective hardening procedure appeared to be that given by the test itself. Consecutive, daily repetition of the test caused a progressive increase in tolerance for the erect position, reaching a peak on the third day. A control sequence of periods of enforced standing, without accompanying chilling, was without substantial hardening effect. The benefit was not maintained in the absence of further, supportive chillings at intervals of not longer than 3 days.

Ascorbic acid. An increased tolerance for the erect position was apparent in rabbits given a single feeding of 300 mg of ascorbic acid 2 hr before the time of test, providing a preliminary chilling had been given not longer than 3 days before. No clear effect from ingested ascorbic acid was apparent in the absence of the reinforcing chilling. As great an improvement was produced by the one preliminary chilling plus ascorbic acid as was produced by 2 preliminary chillings without ascorbic acid. The improvement was transient and usually entirely lost within one to 2 weeks of the time of ascorbic acid

TABLE I.
Reproducibility of Effect of Enforced Standing on Ability of the Rabbit to Warm Up After Chilling; Influence of Infection.

No. of rabbits	Presence of recognized low-grade infection	Preparation*	Avg. temp. rise, °F, during the half hour of standing		Probable error of the difference, °F†
			Test 1	Test 2	
14	+	R	0.85	0.84	0.05
20	—	R	1.39	1.33	.09
20	+	H	1.25	1.24	.05

*R: No preliminary chilling for 4 days or longer, preceding test; H: One preliminary chilling within 3 days of test.
†As computed by Peter's formula (J. W. Mellor, *Higher Math., etc.*, 1926, p. 524).

administration.

The rabbits used were New Zealand White males, between 2.5 and 5.0 kg in weight, with continuous access (excepting during the periods of test) to clover hay, oats, carrot, salt and water. No opportunity for unusual ascorbic acid depletion had been given prior to test nor were unusually low plasma ascorbic acid levels present in those of the group tested for this factor.

Isoascorbic acid. This substance, chemically similar to ascorbic acid but with only a fraction of the latter's antiscorbutic activity,[13] appeared to be practically devoid, also, of effect on sensitivity to gravitational interference with circulation. It was given exactly as the ascorbic acid was given and to the same rabbits, in part before the tests for response to ascorbic acid and in part after.

*Desiccated thyroid.** Feedings of 200 mg per day for 8 days produced a marked decrease in sensitivity to gravitational effect, further decreased by a continuance of the

TABLE II.
Effect of Preliminary Hardening, Ascorbic and Isoascorbic Acids and Desiccated Thyroid.

No. of rabbits	Preparation*	Avg temp. rise in °F during the half hour of forced standing		% change
		Before the indicated preparation	After	
30	H	1.14	1.46	28± 7†
18	H, IA	1.08	1.45	34±11
27	H, AA	1.01	1.77	76±13
23	HH	1.03	1.75	70±15
15	PAA	.92	.77	—17±11
17	T	1.19	1.98	66±14
12	TT	.98	1.86	90±24
17	H, T	1.19	2.28	92±19
12	H, TT	.98	1.99	103±24
13	PT	1.14	1.36	19±11

*H: One preliminary chilling within 3 days of the test.
HH: Two or more chillings during the 3 days preceding test.
IA: Isoascorbic acid, Pfizer, 300 mg by mouth, 2 hr before test.
AA: Ascorbic acid, Pfizer, 300 mg by mouth, 2 hr before test.
T: Desiccated thyroid, Parke-Davis, 200 mg by mouth daily for 8 days.
TT: Desiccated thyroid as indicated under T followed by 100 mg daily for 6 days longer.
PAA: Seven to 14 days after the administration of ascorbic acid:
PT: Seven to 14 days after the discontinuance of the thyroid feedings.
†Probable error of the estimate. See Table I.

[13] Dalmer, O., *Deut. med. Wchnschr.*, 1934, **60**, 1200.

* Trial of this principle was suggested by Broda Barnes who observed instances of improvement in toleration for the erect position in a series of university students given thyroid feedings (personal communication).

feedings for 6 days at a level of 100 mg per day, and by one preliminary chilling on the day prior to test (Table II). Adjuvant hardening was not required for the production of the thyroid effect. Discontinuance of the thyroid feedings was followed by a return toward the original sensitivity to gravitational effect within 5 to 7 days.

Summary. A technic is proposed through which changes in degree of tolerance for the upright position can be followed in the rabbit. Through use of the technic, a minimum tolerance is demonstrated in rabbits harboring depleting infections, and in rabbits softened by maintenance for a week or more in cages too small to permit exercise, and a heightened tolerance following subjection to either a preliminary chilling sequence, a single preliminary chilling followed by ascorbic acid administration, or a sequence of feedings of desiccated thyroid.

13888

Effect of Heparin on Retrograde Blood Flow.

EUGENE LEITER. (Introduced by Claude S. Beck.)

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The use of heparin in the treatment of thrombosis is chiefly prophylactic.[1,2] There is some speculation that thrombi already formed are reduced in size by heparin, but no convincing evidence to that effect. It has also been suggested that heparin may increase the rate of blood flow, by reducing viscosity. If true, this fact would lend further reason for its use in the treatment of occluded vessels, by improving circulation through collaterals. This experiment is intended to show whether retrograde blood flow (*i.e.*, through a collateral circulation bed) is improved by administration of heparin.

Experiments. Dogs and cats were used. Under pentobarbital anesthesia, a femoral artery was ligated and severed. The amount of back-flow in 1-3 min from the distal end of the severed artery was then measured until constant readings were obtained, allowing sufficient time for opening of the femoral collateral circulation. Between readings, the free distal end was kept closed by a clamp, preventing excess loss of blood. Carotid pressure was recorded. Blood pressure was kept as constant as possible. A femoral vein was cannulated for infusions and for injection of heparin. After consistent control readings were obtained, the animals were given single doses of heparin (Liquæmin), the dose varying from 1000 to 3000 Howell cat units per kg. Readings were taken for one-half to 4 hr after heparinization—that is, until the clotting time had returned to normal or at least until the peak of heparinization was well passed.

The method was shown to be sensitive to changes in blood pressure, to blood dilution by saline infusion in excess of 100 cc and to excessive blood loss. Infusion of 200-300 cc of saline in dogs consistently produced a change in the rate of flow, varying from 15% to 30% increase—obviously a factor of dilution and consequent alteration of viscosity. This procedure was utilized at the conclusion of each experiment to check the reliability of the method.

Observations. Experiments were completed on 11 animals, including 9 dogs and 2 cats. In 7 of the 11 experiments, there was no increase of flow after heparinization; in 4 there was an increase of 15% to 30%, attributable to changes in the control conditions.

Conclusion. From the data obtained, hep-

[1] Mason, M. F., *Surgery*, 1939, **5**, 451, 618.

[2] Murray, G., *Surg., Gynec., and Obstet.*, 1941, **72**, 340.

arin has no influence on the rate of retrograde blood flow from the distal end of an occluded artery. Heparin is probably of value in arterial occlusion, not by increasing the rate of flow, but by inhibiting the growth of the thrombus at the site of the occlusion.

13889 P

Endocrine Function of Ovarian Tissue after Growth or Storage *in vitro*.*

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In the course of studies on the effects of anterior pituitary hormones on ovarian tissue grown or stored *in vitro*, it was deemed necessary to develop a technic for determining whether the ability of such tissue to produce estrogen was lost. Accordingly a method was developed for testing the function of ovarian tissue by transplanting it, subsequent to storage or culture, into castrate female rats. The viability of ovarian tissues subjected to a variety of physical factors has been measured by Lipschütz[1] in terms of the hyperfeminization of male guinea pigs after implantation of the tissues on the surface of the kidney. Grafts of thyroid,[2] parathyroid,[2] pancreas,[3] pituitary,[4] and adrenal[5] tissues have been grown in culture prior to implantation in an attempt to acclimatize the tissue to its prospective host, but the routine implantation of cultured endocrine tissue to measure its function has not been utilized previously.

Ovaries from Sprague-Dawley rats, 28 to 32 days of age, were cut into pieces approximately one mm square, maintained *in vitro* under varied conditions, and implanted into the anterior chamber of the eye of adult, castrate females of the Sprague-Dawley strain. Vaginal smears were taken daily and the condition of the implant observed. A smear composed of cornified cells and a few leucocytes was considered a sign of the functional activity of the graft. The possibility of hypertrophied ovarian rests being the source of the vaginal stimulation was checked by removal of the grafts. In every case this procedure was followed by return to a typical castrate vaginal smear.

The ovaries were treated in the following way: (a) cut in human placental serum and implanted directly; (b) maintained *in vitro* for 5 days at 37°C, using the roller tube method of culture;[6] (c) prepared as in (b) but kept at 10°C for 5 days; (d) cut into small pieces, frozen in liquid air, and transplanted immediately or after storage for 2 days at —10°C.

A thick capsule of fibroblasts was observed in the 36-hr-old cultures maintained at 37°C. After implantation vascularization of the grafts by the small vessels of the iris occurred in 2 or 3 days. Rapid growth followed the establishment of a blood supply and in 10 to 14 days the grafts completely filled the anterior chamber. Large follicles and corpora lutea were seen in many of the grafts.

* Supported in part by a grant from the Wisconsin Alumni Research Foundation.

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2 Stone, H. B., Owings, J. C., and Gey, G. O., *Lancet*, 1934, **1**, 625; *Am. J. Surg.*, 1934, **24**, 386; 1934, **100**, 613; *Surg. Gynec. and Obst.*, 1935, **60**, 390.

3 Murray, M. R., and Bradley, C. T., *Am. J. Cancer*, 1935, **25**, 98; Selle, W. A., *Am. J. Physiol.*, 1935, **113**, 118.

4 Haymaker, W., and Anderson, E., *J. Path. and Bacteriol.*, 1936, **42**, 399.

5 Lux, L., Higgins, G. M., and Mann, F. C., *Anat. Rec.*, 1937, **70**, 29; 1937, **67**, 353.

6 Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1933, **17**, 752; 1936, **27**, 45.

TABLE I.
Vaginal Cornification Following Implantation of Ovarian Cultures.

Treatment of ovaries	Host animals: No. with implants	Host animals: Showing vaginal cornification %	Days from implantation to cornification: Avg	Days from implantation to cornification: Range
Direct transfer	8	100	13	6-16
Cultured 2-5 days, 37°C	20	75	14	8-24
" " " 10°C	13	76.9	13	11-17
Frozen quickly in liquid air	14	0	—	—

The results presented in Table I show that the transplanted ovarian tissue which had been maintained at 37°C or 10°C became functional, in terms of vaginal cornification, in approximately the same length of time as the tissue transplanted immediately. Ovarian tissue frozen quickly in liquid air did not produce vaginal cornification during 50 days of observation. Vaginal smears characteristic of estrus were observed in approximately 75% of the animals receiving ovaries maintained both at 37°C and at 10°C, whereas all of the animals receiving ovarian tissue implanted immediately after removal from the animal showed such smears.

The practicability of the test for endocrine function has been demonstrated for ovarian tissue and it is probable that it will be applicable to small pieces of any of the endocrine organs. The anterior chamber of the eye is easily accessible for implantation, observation, and graft removal, and provides an adequate blood supply with relatively little pressure or congestion.

Summary. A method has been described for testing the endocrine function of ovarian tissue after growth in culture at 37°C or storage at 10°C. Such tissue, when transplanted into the anterior chamber of the eyes of castrate female rats, after a latent period of from 8 to 24 days, produced typical estrous type of vaginal smears which compared favorably with the effects produced by transplants made directly after the removal of the tissue from the donor animal. Ovarian tissue frozen quickly in liquid air failed to produce cornification of the vaginal epithelium.

13890

External Field of the Nerve Impulse.

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Since the time of Hermann,[1] the conception of the nerve impulse as a self-propagating electric stimulus has become more and more widely accepted. Within the last few years several experiments have been reported purporting to test the consequences of this view of the impulse as an electrical circuit with two longitudinal resistive components (the transverse ones do not concern us here), one within the core of the axon itself, the other existing outside the core and involving the surrounding media.[2-4]

An additional possible consequence of the local circuit theory concerns the electro-

1 Hermann, L., *Pflüger's Arch.*, 1899, **75**, 574.

2 Hodgkin, A. L., *J. Physiol.*, 1939, **94**, 510.

3 Katz, B., and Schmitt, O. H., *J. Physiol.*, 1939, **96**, 9P-10P.

4 Katz, B., and Schmitt, O. H., *J. Physiol.*, 1940, **97**, 471.

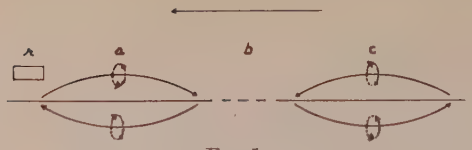

FIG. 1.
r represents cross-section of a toroidal coil.

magnetic properties of the field surrounding the region occupied by the advancing impulse. If the theory is true, the magnetic field surrounding the external component of the circuit should be oriented in a manner which is opposed to that of the internal component, with the result that, owing to the extreme smallness of the core's diameter, almost complete cancellation of the magnetic fields will result. (Fig. 1). If, therefore, a suitable detecting instrument is placed outside the nerve itself, no magnetic field should be observed.

Apparatus. The detecting instrument was a toroidal coil made of thirty turns of No. 28 wire wound longitudinally about a cylindrical core made of a stack of Numetal plates with a 3/32″ hole through the center. The coil was 1 cm long. This was imbedded in a "Lucite" cylinder (2 cm in diameter) and carefully insulated with "Amphenol dope" in such wise that there could be no leakage between the contents within the aperture and the wire. The coil was tested for electrical leakage before and after each experiment. The coil leads were inducted into a matching transformer whose output was fed into an amplifier, unbalanced to ground. The recording instrument was a commercial Dumont oscillograph.

By means of this arrangement (Fig. 2) it was possible to use the toroidal coil as a magnetic detecting device. By grounding the primary of the transformer, any purely electrical coupling between the toroid and the recording instrument would be obviated and hence any disturbance transmitted to the oscillograph would indicate an induced EMF in the pick-up coil.

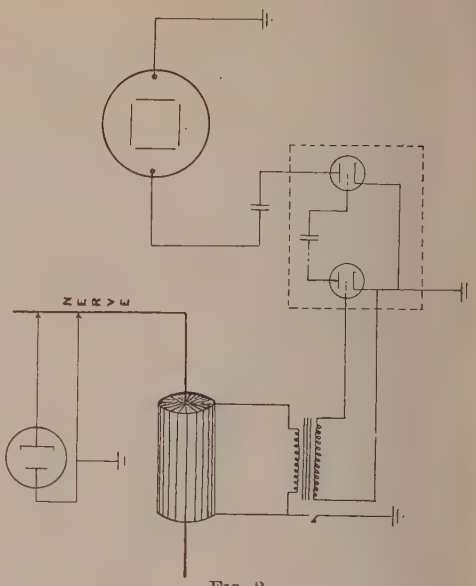

FIG. 2.

The pick-up system possessed a sensitivity permitting the detection of the magnetic field of a .10 μ A current at 500 cycles sent through the center of the coil.

The frog's sciatic was used.

Results. Many repetitions of the experiment gave negative results.

Experiments were performed wherein the nerve preparation and the toroidal coil were submerged in saline solution. In this case, the conductive saline solution surrounding the nerve could be expected to act as a low resistance shunt (Hodgkin), drawing off a portion of the external component of the bioelectric current and thus provide for a greater physical separation between the internal path of the current and a portion of the external path. This would have the effect of diminishing in some degree the interaction between the oppositely oriented magnetic fields.

The results in this case, however, were also negative.

13891

Inhibitory Effect of Heparin upon Histamine Release by Trypsin, Antigen, and Proteose.*

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Anaphylactic, trypsin, and peptone shock are closely related phenomena. The prominent symptoms are similar and the intravenous injection of antigen, trypsin, or proteose into intact animals has been shown to result in the liberation of significant amounts of histamine.[1-3] It has also been shown that the addition of antigen, trypsin, or proteose to rabbits' blood (rendered incoagulable with heparin) leads to the release of histamine from cells to plasma.[4-9] It is possible that the mechanisms of the release are the same in each case. A study of the effect of various agents in inhibiting or augmenting the release of histamine by the above substances suggests itself as a means of determining whether similar mechanisms are involved. Inasmuch as rabbits' blood can be divided into samples which can be considered identical in every respect, this tissue seems to provide an ideal medium for such studies.

It has been shown that heparin is capable of inhibiting the proteolysis of various substrates by trypsin,[10,11] and we have found that heparin antagonizes the toxic effects of trypsin in intact animals.[12] Consequently the present experiments were performed to determine whether heparin has an inhibitory effect upon the release of histamine by trypsin, and if so, to examine the effect of heparin upon the release of histamine by antigen and by proteose.

Experimental. Blood was obtained from unanesthetized rabbits by cardiac puncture. Sufficient heparin‡ was added to this blood to prevent clotting, a concentration of 0.02%. Each blood sample was divided into 3 tubes. Saline was added to the first tube; specific antigen, trypsin, or proteose was added to the second tube; and specific antigen, trypsin, or proteose plus additional heparin, a final concentration of 0.12%, was added to the third tube. The tubes were carefully inverted a few times and then immediately centrifuged for 5 min in an angle centrifuge. The supernatant plasma was pipetted off and assayed for its histamine equivalent content against standard histamine solutions on either the guinea pig intestine or the blood pressure of an etherized-atropinized cat. The plasma samples were extracted in most cases by Code's modification of the method

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† Guggenheim Fellow, Instituto Biologico, Sao Paulo, Brazil.

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5 Dragstedt, C. A., Ramirez de Arellano, M., and Lawton, A. H., *Science*, 1940, **91**, 617.

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9 Gotzl, F. R., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1942, **74**, 33.

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11 Glazko, A. J., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 43.

12 Dragstedt, C. A., Wells, J. A., and Rocha e Silva, M., *Fed. Proc.*, 1942, **1**, 149.

‡ Connaught, Lederle, and Roche-Organon heparin have all been employed with equal results.

TABLE I.
Effect of Excess Heparin upon the Release of Histamine from Cells to Plasma in Rabbit's Blood.

Rabbit No.	Histamine releasing agent	Plasma histamine (micrograms of histamine base/cc)		
		I	II	III
1	Trypsin	0.51	1.45	0.51
2	"	0.20	0.72	0.29
3	"	0.09	0.42	0.29
4	"	0.00	0.72	0.36
5	"	0.27	1.52	1.30
1	Proteose	0.45	1.20	0.21
2	"	0.29	0.80	0.27
3	"	0.82	1.60	0.80
4	"	1.33	2.66	<0.53
5	"	0.51	0.72	<0.26
6	"	1.54	1.86	0.52
7	"	0.40	3.09	0.24
8	"	1.54	3.09	0.53
1	Antigen	0.27	4.26	0.48
2	"	2.02	3.41	2.24
3	"	3.52	7.24	2.66
4	"	2.39	3.46	1.33

Column I: Plasma obtained from weakly heparinized bloods (0.02%).
Column II: Plasma obtained from weakly heparinized bloods (0.02%) to which a histamine releasing agent: antigen (egg white, 4.7 mg/cc of blood), peptone (Bacto-protone, 4.7 mg/cc of blood), or trypsin (crystalline trypsin, 0.4-0.6 mg/cc of blood) was added.
Column III: Plasma obtained from strongly heparinized bloods (0.12%) to which identical amounts of the above releasing agents were added.

of Barsoum and Gaddum.[13] In a few cases the unaltered plasmas were tested directly on the atropinized cat.

For the anaphylactic experiments, rabbits were sensitized to powdered egg white by repeated injections and a sufficient quantity of 10% solution was added to the blood to make the final concentration 4.7 mg/cc. For the peptone experiments, a sufficient quantity of a 10% solution of Bacto-Protone (acidified, shaken with permutit, filtered and neutralized) was added to the blood to make the final concentration 4.7 mg/cc. For the trypsin experiments, sufficient crystalline trypsin (Plaut) was added to the blood to make the final concentration 0.4-0.6 mg/cc. The results are shown in the accompanying table.

Discussion. It is apparent that increased amounts of heparin inhibit the release of histamine from cells to plasma whether this is brought about by trypsin, proteose, or specific antigen. The results thus far do not permit a precise statement as to the concentration of heparin required to exhibit this inhibiting effect, but they indicate that it must be considerably in excess of that required to prevent coagulation.

There are a number of reports in the literature regarding the effect of heparin upon anaphylactic shock in intact animals. Some workers have obtained marked inhibiting effects while others have obtained none. It seems probable from the present results that the negative experiments may have been due to the employment of an ineffective concentration of heparin.

The parallel effect of heparin in the 3 cases suggests that the mechanisms of histamine release may be the same in each case, that is to say, it is possible that some protease activity may be responsible for the histamine release in anaphylactic and peptone shock.

[13] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

13892

Occurrence of Brain Hemorrhages in Choline-Deficient Rats.*

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The importance of choline in animal nutrition has been the object of much recent interest. In young rats reared on diets adequate in dietary essentials other than choline, hemorrhagic degeneration of the kidney, and, at times, intraocular hemorrhages are characteristic pathologic findings.[1,2] Symptoms of involvement of the nervous system, apparently caused by choline deficiency, have been observed by Sure[3] and by György and Goldblatt.[2] In young rats of mothers reared on choline-deficient diets, these authors described a paralysis which develops at about the thirteenth day and is followed by death in 3-5 days if choline is not added to the diet. The pathology underlying this type of paralysis has not yet been investigated. It is the purpose of this note to put on record the changes in the nervous system occurring in young rats of mothers reared on a diet low in choline and high in cystine.

Experimental. Five female rats were given the following diet from about the eighth day of pregnancy on:

Purified Casein Smaco	25
Cystine	0.5
Dextrine	58.5
Agar	2
Crisco	10
Salt mixture	4

In addition, daily doses of 100 γ of thiamine, riboflavine, pyridoxine and calcium pantothenate and 6 mg of nicotinic acid were given. Two drops of haliver oil were added weekly to the diet. As controls two female rats were reared on the same diet without cystine and with the addition of 100 mg of choline per 100 g of food.

No significant differences were noted between the young born from deficient and normal rats until the 10th-12th day when cessation of growth was noted in the deficient group. About 50% of the rats died at this stage without showing symptoms of involvement of the nervous system; the others developed paralysis of the limbs. This was often accompanied by tremors of the head and the whole body and occasionally by violent paroxysms of jerking movements similar to generalized convulsions. Death followed in 3-5 days.

The nervous systems of 5 rats which had shown conspicuous neurological symptoms were examined histologically by the common methods of neuropathologic technic. The most striking alteration consisted of extensive hemorrhagic lesions of the cerebellum. From one-third to half of the cerebellar lamellæ appeared involved (Fig. 1). Hemorrhage was most extensive in the central part of the lamella, often occupying the entire white matter. Myelin sheaths and axis-cylinders were destroyed. In the granular layer only scattered foci of hemorrhage were present which appeared roundish in configuration. The layer of Purkinje cells was relatively well preserved whereas in the molecular layer extensive hemorrhages were present involving particularly its external portion. Blood was present also in the subpial spaces. The central medullary substance and the nucleus dentatus were intact.

By the Pickworth stain for blood vessels, no hemorrhages were demonstrable in the mesencephalon and diencephalon. In the cerebral cortex small foci of hemorrhages scattered throughout the gray matter and the subcortical white matter were found in 2 cases.

In every instance the hemorrhages appeared to be recent in character. No reaction of glia or connective tissue was observed.

* Aided by a grant from the Penrose Fund.

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2 György, P., and Goldblatt, H., *J. Exp. Med.*, 1940, **72**, 1.

3 Sure, B., *J. Nutrition*, 1940, **19**, 71.

FIG. 1.
Showing extensive hemorrhagic lesions of the cerebellum. Nissl stain. Low power.

Alterations other than hemorrhagic appeared of little significance. Frequently there were acute degenerative lesions of the neuron cells diffusely distributed. The peripheral nerves showed no pathology.

Comment. Hemorrhagic lesions within the nervous system have been previously described in thiamine deficient rats[4,5] and pigeons.[6,7] However, these hemorrhages are apparently different from those found in choline deficiency. In thiamine deficiency the hemorrhagic lesions are localized in the vestibular nuclei, the thalamus, hypothalamus, periventricular region of the third ventricle, oculomotor nucleus, culliculi and, only occasionally, the cerebellum, whereas in choline deficiency the cerebellum and, occasionally, the cerebral cortex are involved, the other regions being intact. Moreover, in thiamine deficiency the hemorrhages are circumscribed and small, while in choline deficiency they are diffuse and massive. Finally, in thiamine deficiency the hemorrhages are preceded by, and apparently secondary to, degenerative changes in neighboring nervous parenchyma[5,7] while these primary parenchymatous changes have not been observed in choline deficiency.

Hemorrhagic lesions of the cerebellum have been previously recorded in Vitamin E-deficient chicks,[8] but they appear to be preceded or accompanied by degeneration and necrosis of the nervous tissue and thrombosis of the blood vessels, features which were not observed in the present experiments.

Although the causes of hemorrhage in the brain of choline-deficient rats are unknown, the lesions of the blood vessels appear primary in character and similar in nature to those encountered in the kidneys of rats reared on similarly deficient diets.

Summary. Hemorrhagic lesions of the

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7 Prados, M., and Swank, R. L., *Arch. Neur. and Psychiat.*, 1942, **47**, 626.
8 Wolf, A., and Pappenheimer, A. M., *J. Exp. Med.*, 1931, **54**, 399.

cerebellum, occurring in young rats of mothers reared on a diet containing a high percentage of cystine and deficient in choline, are described and briefly compared to the brain hemorrhages observed in other vitamin deficiencies.

13893

Quinones as Blood Pressure Reducing Agents in Hypertensive Rats.*

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Introduction. Following the work of Holtz,[1] Bing and his co-workers[2,3] have demonstrated that experimental renal hypertension can be produced by the injection of amino acids into the ischemic kidney of cats. This work has provided a basis for the hypothesis that the hypertension resulting from an interference with the blood supply to the kidney may be due to faulty deamination of certain amino acids. This process is catalyzed by amine oxidase and requires the presence of oxygen. Under anaerobic conditions, however, such as may exist in renal ischemia, decarboxylation without subsequent deamination would occur, thus leading to the formation of pressor amines. An accumulation of these substances would tend to elevate the blood pressure.

It has been shown that certain pressor amines can be inactivated by quinone precursors.[4,5] The present study deals with the effect of certain quinones on the blood pressure of rats with experimental renal hypertension.†

Method. Young male rats weighing 150-200 g were used. Hypertension was produced by wrapping one or both kidneys in cellophane.[6] Blood pressures were recorded in the tail using the method of Williams, Harrison and Grollman.[7] The systolic blood pressures of over 600 normal rats tested by this method in our laboratory ranged between 100 and 140 mm of mercury. The blood pressure of hypertensive rats was generally 40 to 80 mm of mercury above the preoperative level. Rats were considered suitable for testing when the blood pressure level had been elevated and stable for a period of at least one month. Daily readings were made for one week prior to, during and immediately following administration of the agent to be tested. Nine compounds were tested by the subcutaneous and 5 by the oral route. When used orally a weighed sample of the compound was mixed daily with a submaximal quantity of the food which the animal had been accustomed to consume. Thus the daily dosage in feeding experiments was approximate and not exact. Quinones which proved effective in reducing the blood pressure in hypertensive animals were tested also on rats with normal blood pressure.

Results. Four of the 10 compounds tested were effective in lowering the blood pressure of hypertensive rats. As will be seen in

* Several of the compounds used in this work were secured through the courtesy of the Wallerstein Laboratories, to whom we are deeply indebted.

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4 Soloway, S., and Oster, K. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 108.

5 Oster, K. A., *Nature*, 1942, **150**, 289.

† Similar experiments on hypertensive dogs are being conducted.

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TABLE I.
Effect of Various Quinones on the Blood Pressure of Hypertensive Rats.

Quinone tested	Method of administration	Medium	Effect	No. of rats used	Avg initial B.P. level Mm Hg	During administration: Avg lowest B.P. Mm Hg	During administration: Avg max. change Mm Hg	Avg No. of days before change	Daily dose mg
2,5 Dimethyl paraquinone*	subc.	saline	anti-pressor	8	191	124	67	3	1-2
2,5 " "	"	oil	"	4	186	130	56	5	1-4
2,5 " "	oral	food	"	6	186	121	65	7	25-100
2 Methyl 5 isopropyl paraquinone†	subc.	saline	"	12	188	135	53	2	2-10
2 " 5 " "	"	oil	"	29	185	130	55	5	10-40
2 " 5 " "	oral	food	"	13	191	136	55	4	100-200
2 " 5 " "	"	oil	"	15	185	135	50	4	8-50
Sodium rhodizonate	subc.	saline	"	12	190	129	61	3	6-12
Trimethyl paraquinone	"	"	"	12	188	136	52	3	2-8
Triquinoyl	"	"	0	6	186	161	25	—	10-30
4,5 Dimethyl orthoquinone	"	"	0	12	194	180	14	—	5
2,5 Dihydroxy paraquinone	"	"	0	4	206	180	26	—	5
2,5 Dimethyoxy paraquinone	oral	food	0	4	220	206	14	—	154
Sodium chloranilate	subc.	saline	0	4	177	169	8	—	2
" "	oral	food	0	4	176	171	5	—	175
β-Hydroxy 1,4 naphthaquinone	subc.	saline	0	4	203	189	14	—	2
β " 1,4 "	oral	food	0	4	202	192	10	—	470

*Xyloquinone.
†Thymoquinone.
Subc. = subcutaneous.

Table I, the 4 effective substances were (1) 2,5 dimethyl paraquinone (Xyloquinone), (2) 2 methyl 5 isopropyl paraquinone (Thymoquinone), (3) sodium rhodizonate and (4) trimethyl paraquinone. In most cases in which a depressor effect was obtained, the blood pressure of the hypertensive rats was reduced to the normal range and on discontinuing the administration of the compound the hypertension returned, but usually not quite to the previous hypertensive level. This depressor effect could be produced repeatedly on the same animal, the blood pressure returning to a high level each time after discontinuing the medication. The 6 other compounds tested were ineffective in reducing the blood pressures by amounts which we regarded as significant.

Table I summarizes the results of experiments on over 100 rats, many of which were used in more than one experiment. It will be seen that only such substances were considered anti-pressor as produced (1) a fall to at least 150 mm of mercury, and (2) an average maximum drop of at least 30 mm.

Fig. 1 illustrates typical averaged results with one of the effective compounds, xyloquinone, injected subcutaneously.

It is noteworthy that the 4 effective compounds did not influence the blood pressure of normal non-hypertensive rats.

One of the effective compounds, trimethyl paraquinone, sometimes gave toxic symptoms (methemoglobinemia), so that further ex-

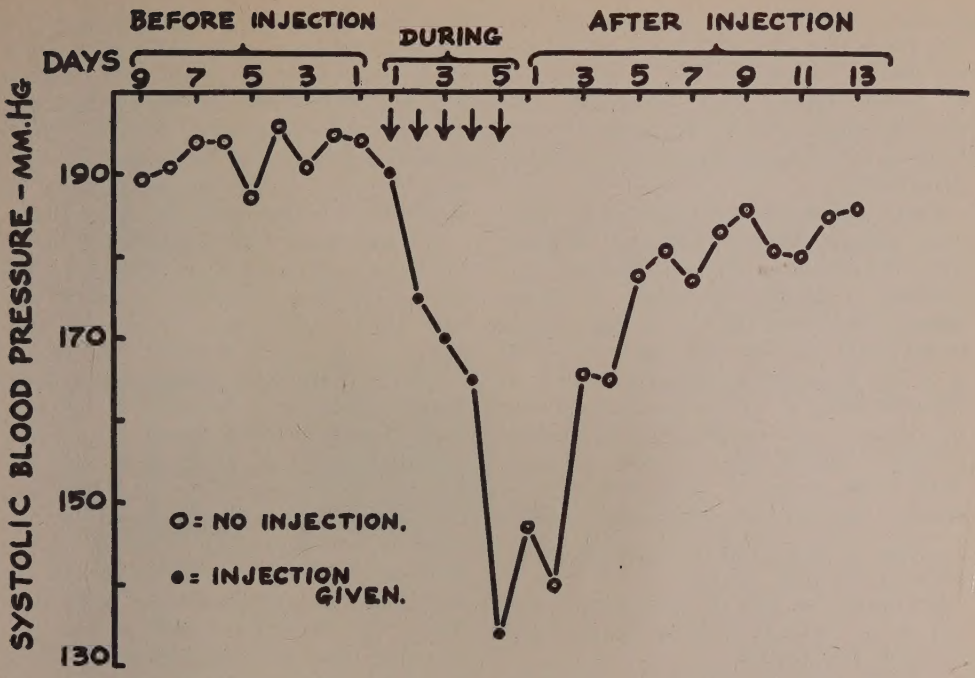

FIG. 1.
Anti-pressor effect of subcutaneous injection of xyloquinone in saline. Results represent averaged blood pressures of 4 rats. Dosages were from 1-4 mg for 5 successive days.

periments with this substance were discontinued. In the case of one ineffective substance, β-hydroxy 1,4 naphthaquinone, oral administration of even such a huge dose as 470 mg daily for 4 days produced no effect on the hypertension, but produced orange pigmentation of the skin and mucosa of the rats.

In several of the experiments the temperature of the rats was measured before and during the administration of quinones, and in none was there a significant change in temperature. In several instances, however, with large doses of quinones, death occurred preceded by rapid decline in blood pressure.

When the compounds xyloquinone or thymoquinone were administered with ground-up rat food, the rats lost weight and the blood pressures fell, but control experiments convinced us that the drop in blood pressure was not associated with the loss of weight as such, but was due to the compounds administered. The solubility of the preparations of quinones used was of varying degree, and so some had to be injected as suspensions.

Experiments were performed to determine the stability in oil of one of the effective compounds, *i.e.*, thymoquinone. After solution in olive oil it was allowed to stand in the refrigerator for 6 weeks; on being then tested experimentally the solution was as effective as it had been originally. Chemically, the effective compounds are stable if kept in dry containers in the dark.

Discussion of Results. Three of the 4 effective compounds (xyloquinone, thymoquinone and trimethylquinone) are paraquinones, while the fourth (sodium rhodizonate) is a diorthoquinone. The paraquinones in general have the advantage of greater stability in saline solution and thus are to be preferred to the orthoquinones.

It is not clear at the present time just why some of the quinones are effective while others are not. Too few compounds have been tested to permit correlation of anti-

pressor properties with chemical structure. The mechanism of action of the quinones is not clear. The inactivation or destruction of pressor amines may conceivably take place by conjugation, by formation of Schiff bases or by acting as catalysts for oxidative deamination.

Great caution must be employed in evaluating changes in blood pressure resulting from therapeutic agents administered parenterally. Striking reductions in blood pressure have been reported in man and in experimental animals following the use of a variety of non-specific measures such as the administration of pyrogens,[8] inactivated tyrosinase,[9] and implantation of foreign protein.[10] All of these reactions are associated with fever or local inflammatory changes. In this study slight local reactions occurred with some, but not with all of the effective quinones used. Equally severe local inflammatory reactions were observed with most of the other non-effective quinones. Febrile reactions were not observed. Moreover, the preparations were effective when used by mouth so that the effect cannot be attributed to a non-specific pyrogenic action.

The fact that the blood pressure of normal non-hypertensive rats is not affected by potent anti-pressor quinones, suggests that their action is not that of a general vasodilator.

Summary. 1. Three paraquinones and one diorthoquinone were found to have well-defined blood pressure reducing properties in hypertensive rats. These compounds are 2,5 dimethyl paraquinone (xyloquinone), 2 methyl 5 isopropyl paraquinone (thymoquinone), trimethyl paraquinone and sodium rhodizonate. 2. This depressor effect is not associated with a febrile reaction and can be elicited by the oral as well as the subcutaneous route of administration. 3. These compounds have no effect on the blood pressure of normal non-hypertensive rats.

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